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BIOLOGIC VARIABLES ASSOCIATED WITH *CUMMULUS* OOCYTE COMPLEX RECOVERY USING FOLLICULAR ASPIRATION

Viana, J.H.M.[1]; Bols, P.E.J.[2]

[1]Embrapa Gado de Leite, Juiz de Fora, MG, 36038-330 Brasil
[2]University of Antwerp, Universiteitsplein 1, Gebouw U, 2610 Wilrijk, Belgium
jhmviana@cnpgl.embrapa.br

Introduction

The ultrasound guided follicular aspiration (OPU) was developed in the 80's decade to attend the needs in obtaining the cummulus–oocyte complex (COCs) using a less traumatic procedure than the surgical approach or laparoscopy. The ultrasound guided follicular aspiration was later adapted for veterinarian use, being rapidly known as the elective technique to recover COCs (Galli et al., 2001). Among the recognized advantages, we can cite: its low invasive aspect, its suitable application regardless hormonal stimulation, and the possibility of being used in any phase of the estrous cycle, during pre-puberty or early gestation. In addition, it overcame the main obstacle encountered by the commercial use of *in vitro* fertilization (IVF), which was the recovery of COCs from live donor cows, which has been considered the best alternative to classic superovulation programs for embryo production (Bousquet et al., 1999). The low efficiency of this system, initially used, limited its commercial application on follicular aspiration during the 80's and 90's decades. Only animals with a high genetic value and acquired fertility problems, in addition to low response upon superovulation, were submitted to the procedure. However, in the past years, this picture has been changed and characterized by the intensive use of donors reproductively active and by the increase on *in vitro* embryo production worldwide, principally in South America and Asia (Thibier, 2004). This evolution was remarkable, specially when considering the blastocyst production rates obtained by different research groups, which remained around 25 and 40%. The increase on numbers and/or quality of COCs recovered and submitted to *in vitro* fertilization can overcome the partially low efficiency of the available culture systems. Historically, two important aspects have been used to optimize the COCs recovery: (1) studies on related mechanical and physical factors and consequently improvement of aspiration equipment; (2) study and control of factors related to development and maturation of intra-ovarian follicles and oocytes (Bols et al., 1997). The first aspect was the most explored, leading to the development or adaptation of numerous options on follicular aspiration systems. It can be assumed that the number and quality of COCs submitted to culture are responsible for the success of *in vitro* embryo-production when considering the results obtained in the lab phases.

Availability of follicles for aspiration

The physical characteristic of an ultrasound equipment (image and resolution) allows to identify and consequently, only aspirates antral-phase follicles with diameters superior to 2 and 3 mm. Those

follicles are representative of a small fraction of the total ovarian follicles. The number of follicles at a determined moment are known to be dependent on the ovarian reserves mobilization (I), follicular development at initial phases, and their association with the donor metabolic and endocrinology status (II), and the growth dynamics during the antral phase (III).

Maximization of antral follicles, regarding number and quality are the main strategies to improve recovery of COCs for IVF.

I-Ovarian Reserves

In all mammal females, the oocyte reserves are established during the fetal stage, being gradually mobilized during the reproductive life (Van der Hurk, 2005). Despite of this being considered one of the most important characteristics in determining the potential of a female as a donor (regarding oocyte or embryo yielding), a practical procedure is lacking for *in vivo* evaluation of those reserves. In human medicine, indirect tests for ovarian reserves are established, based on FSH/ Inhibin blood concentrations, even though, with limited precision and different objectives. A relationship seems to exist between size of primordial follicle reserves and proportion of follicles that start their development (Hirshfield, 1994). Thus, the number of follicles at the antral-phase, which are observed during estrous cycle can, at least, partially represent the primordial follicle reserves (Gülekli et al., 1999). Significant variations on follicle numbers under development in the ovaries are reported among individuals and breeds (Boni et al., 1997). And the ovarian reserves could be the key in explaining the differences encountered in ET/IVF results from determined genetic groups. However, this relationship can be less evident in production animals due to different factors that may interfering with the process of follicular recruitment, such as: metabolic balance, body score condition, environmental conditions, and others.

II. Follicular recruitment

Unfortunately, the factors influencing the initial stages of follicular development are less known (Fortune, 2003). The initial phases of follicular growth are not dependent upon hypophyseal hormone, and they are controlled by intra-ovarian factors. Some of factors those can present a positive action (IGF-I, EGF, TGFα, bFGF, GDF9, BMP15, ativina, IL 1β, NO) or an atretic action (TNFα, folistatin, androgens, free radicals, IL6) controlling follicular survival and development (Erickson & Shimasaki, 2001). The complex interactions between those factors, and the autocrine and paracrine nature of their action makes extremely complex to manipulate the initial phases of follicular growth. A relatively successful approach was the use of BST in cows increasing the number of follicles under growth. This effect has been attributed to the IGF-1 increased concentrations in the ovary. The use of BST was proposed as an alternative in maximizing the results obtained by follicular aspiration (Buratini Jr., 2000). The results obtained by this strategy and by others, which include indirect manipulation of early follicular growth phases, as an induction of positive energy balance, are inconsistent and limited by the ovarian reserves of a donor cows.

III - Follicular dynamics

Once the antral phase has been achieved, known factors controlling follicular growth can be manipulated. The number of follicles during the antral phase is variable throughout the estrous cycle, due to follicular growth pattern encountered in the bovine (Driancourt, 2001). Each follicular wave is characterized by a group of follicles measuring 2 to 4 mm in diameter, when competence is acquired by the oocytes, which are then able to undergo maturation, meiosis and further *in vitro* embryo development. At this phase, the follicular dominance is the main factor influencing the quality of developing follicles. The follicular dominance phenomenon, mechanism of ovulation rates specie-specific, is based on FSH release inhibition by estradiol secretion, inhibin, and follicular dominance (Crowe, 1999). Thus, the presence of a dominant follicle interferes with the number and quality of available follicles submitted to superovulation (Bungartz & Niemann, 1994) and follicular aspiration. The COCs collected at an early phase of the estrous cycle (during the onset of follicular wave), after wave synchronization or after mechanical removal of dominant follicle has shown to result in a higher number of COCs and /or in a higher potential of *in vitro* development (Machatková et al., 2000). The reduction of interval between subsequent collections has been used to minimize the follicular dominance effect (Petyim et al., 2003). However, the frequent removal of developing follicles in absence of a corpus luteum can lead to changes on hormonal and follicular growth patterns in donor cows (Viana et al., 2004).

An alternative approach is the FSH increased by the use of exogenous gonadotrophins or immunization against inhibin (Blondin et al., 2002; Viana et al., 2003). Despite a positive effect on quality and number of obtained COCs, this strategy presents some of the disadvantages encountered at the conventional superovulation programs, particularly regarding necessary intervals between treatments.

Conclusion

The success obtained from in vitro produced embryos can be largely attributed to the number and quality of COCs used. However, the mechanism evaluation and follicular growth manipulation remain limited to the antral phase. The advances on the effective use of gamete reserves depend upon a better knowledge regarding primordial follicle mobilization mechanisms.

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Leroy J.L.M.R., Bols P.E.J., Opsomer G., Van Soom A. & de Kruif, A. 2005. **Ovum pick-up as the ideal technique to study the intrafollicular environment and oocyte quality in high-yielding dairy cows**. *Acta Scientiae Veterinariae*. 33 (Suppl 1): 5-18.

OVUM PICK-UP AS THE IDEAL TECHNIQUE TO STUDY THE INTRAFOLLICULAR ENVIRONMENT AND OOCYTE QUALITY IN HIGH-YIELDING DAIRY COWS

Leroy, J.L.M.R.[1]; Bols, P.E.J.[2]; Opsomer, G.[1]; Van Soom, A.[1]; Kruif, A.[1]

[1] Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [2]Laboratory for Veterinary Physiology, Departement of Veterinary Medicine, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium. lo.leroy@ugent.br, Tel: ++3292647548 Fax: ++3292647534

ABSTRACT

Fertility is declining in high-yielding dairy cows. This multifactorial problem has been associated with disruptions in the signalling in the hypothalamus-hypophysis-ovarium-axis. It is only recently that attention is paid to oocyte quality and its intrafollicular environment and the ovum pick-up technique proved to be an ideal tool to study these two factors. In this way it became possible to investigate the role of the oocyte and its environment in the pathogenesis of the reduced fertility in modern dairy cows. This paper is a state of the art in that particular research field.
Keywords: high-yielding dairy cow, ovum pick-up, oocyte quality, follicular fluid, reduced fertility

INTRODUCTION

It is well documented in numerous studies world wide that dairy cow fertility has been declining since several decades. This phenomenon has been associated with increased lactational yields (Lean et al., 1989; Royal et al., 2000; Lucy, 2001; Butler, 2003; Lopez-Gatius, 2003; Bousquet et al., 2004; Mee et al., 2004). A significant genetic progress coupled with an improved nutritional management made it possible for dairy farmers to realise enormous milk yields. However, to keep it economically efficient, modern high-yielding dairy cows should conceive within the first three months after parturition, which is metabolically spoken the most demanding period.

Shortly after giving birth, there is a rapid increase in milk production and thus in energy loss, which is impossible to be covered by a sufficient energy uptake through feed (Rukkwamsuk et al., 1999). Especially overconditioned cows display a reduced appetite, a subsequent increased lipid mobilisation and liver triglyceride infiltration, resulting in high plasma ketone levels (Rukkwamsuk et al., 1999). The rapid mobilisation of body reserves, reflected in the loss of body condition score (up to 10% of calving body weight), may aggravate depressed appetite (Macmillan et al., 1998; Vernon 2002). Primiparous cows typically show an even more negative energy balance (NEB) since they still need extra energy for body growth (Cavestany et al., 2005). It has been shown that such metabolic stress, together with several endocrine malfunctions, is hard to reconcile with a satisfying reproductive performance. Biologically spoken, it makes sense to favour milk production over fertility, which is referred to as nutrient prioritization (Lucy, 2003). It is better to invest limited resources in the survival of the current offspring (i.e. milk production) than gamble on the health and survival of those that are

yet to be conceived and born (i.e. fertility) (Silvia, 2003).

Reproductive failure is certainly a multifactorial problem in which the amount of milk production as such only plays a minor role compared with the importance of NEB, body condition score (BCS) or postpartum (pp) diseases (Loeffler et al., 1999; de Vries and Veerkamp, 2000; Snijders et al., 2000; Lucy, 2001). Daily milk yield is not an appropriate indicator of NEB because feed intake and management can confound a possible association between yield and energy balance (Villa-Godoy et al., 1988; Macmillan et al., 1998; de Vries and Veerkamp, 2000; Kruip et al., 2000). Also the high energy and protein rations typically fed to high–yielding dairy cows to sustain milk production together with the increased herd size are factors that have been associated with the disappointing fertility outcomes (Butler, 1998; Lucy, 2001; Fahey et al., 2002; Lucy, 2003). Finally, the genetic selection for high milk production as such may also be a cause of reduced fertility as has been suggested by Snijders et al. (2000, 2001). Taken together, these points emphasize the uncompromising relation between high milk production and fertility.

The pathogenesis of this declined fertility is complex and has been studied already in detail. The aim of this paper is to focus on the importance of oocyte quality in the widely reported reduced capability of getting pregnant. This oocyte is growing and maturing in the follicular environment. Is the oocyte well isolated from all these metabolic and endocrine disruptions or is the vulnerable oocyte really in danger? Information on the composition of the follicular environment and on oocyte quality in relation to the problem of reduced fertility in high-yielding dairy cows is very scarce. The ovum pick-up (OPU) technique proved to be a suitable technique to find answers to all these questions.

MULTIPLE PATHWAYS TO SUBFERTILITY

Specific pathways that lead to disturbed reproduction in metabolically compromised pp dairy cows have been investigated intensively (reviewed by Butler, 2003). Much of the effort has focused on alterations in endocrine signalling (hypothalamus-hypophysis-ovarium axis) and ovarian dysfunction expressed in reduced oestrous symptoms or anoestrus, cyst formation, delayed first ovulation, and prolonged calving to first insemination intervals (Harrison et al., 1990; Opsomer et al., 1998; Beam and Butler, 1999; de Vries and Veerkamp, 2000; Diskin et al., 2003; Vanholder et al., 2003; Lopez et al., 2004). The very final and somewhat more reliable fertility outcome parameter is conception rate, defined as the proportion of cows declared pregnant after AI. As has been reviewed by Bousquet et al. (2004), the success rate of an AI showed a dramatic drop in almost all countries housing high-yielding dairy cows. The concept ‘conception rate’ is closely related to oocyte and thus embryo quality.

It is only recently, however, that few studies began to focus on the oocyte and subsequent embryo quality which may be important factors for conception rate and hence fertility (Boland et al., 2001). Oocytes and embryos are highly sensitive to disruption in their environment by metabolic, dietary or other causes, thereby having fatal consequences for the final fertility outcome (McEvoy et al., 2001). O’Callaghan and Boland (1999) suggested already that the observed decline in fertility in high producing dairy cattle is mostly a problem of bad oocyte- and hence embryo quality, rather than being an endocrine disruption. Very recently, this has been confirmed by Horan et al. (2005). More and more early embryonic

mortality is proposed to be a significant cause of reproductive failure in ruminants (Dunne et al., 1999; Mann and Lamming, 2001; Bilodeau-Goeseels and Kastelic, 2003). Whether this problem originates from bad oocyte quality and/or from a changed microenvironment of the embryo in the fallopian tube and uterus, is not known (Boland et al., 2001). It is in this field that the OPU technique can be of use. First of all it can be applied to collect oocytes in a more or less non-invasive way to study their intrinsic quality. Secondly, it is an ideal tool to lay open and analyse the intrafollicular environment. Both applications will be addressed extensively in the following paragraphs.

OPU TECHNIQUE TO STUDY THE OOCYTE QUALITY IN HIGH-YIELDING DAIRY COWS

The ultimate test for oocyte quality is its ability to be fertilized and to develop to the blastocyst stage and finally to establish pregnancy which results in a living offspring (Lonergan et al., 2001). Unfortunately, it is practically impossible to transfer all evaluated oocytes in living recipients to check for pregnancy. Therefore, we are obliged to use other parameters, which are said to be well correlated with the actual oocyte quality as described above. The most reliable and commonly used parameter is the oocyte's developmental competence in vitro and more specifically the timing of first cleavage (Van Soom et al., 1992). This is related to the polyadenylation status of several developmentally important maternal gene transcripts (Brevini-Gandolfi et al., 2000). The more, other parameters evaluating the proper oocyte are routinely used such as the morphological appearance of cumulus investment and ooplasm (de Loos et al., 1989; Hawk and Wall, 1994), lipid content (Leroy et al., 2005), ultrastructural evaluation of the nuclear stage and ooplasma (Revah and Butler, 1996; Yaakub et al., 1997; O'Callaghan et al., 2000), gene transcripts (Wrynzeki et al., 2000) and presence of apoptosis (Yuan et al., 2005).

OPU is the ideal technique to collect the oocytes from living animals to study these morphological and genetic quality parameters or to investigate their developmental competence. For this purpose the OPU protocol was optimized by Bols (1997).

An important question is now: Is the intrinsic quality of the oocyte compromised in these high-yielding dairy cows? And consequently, is an altered quality of the female gametes an important factor in the pathogenesis of subfertility? Recent studies confirmed this hypothesis by using the OPU technique. Snijders et al. (2000) studied the in vitro developmental competence of oocytes from dairy cows with a high and moderate genetic merit for milk production. Oocytes from modern high genetic merit cows yielded a significantly lower number of blastocysts in vitro, irrespective of milk production as such. Walters et al. (2002) observed in first and second parity cows an increase of the morphological oocyte quality between day 30-70 pp and a clear decrease thereafter. Third parity cows, however, displayed a linearly improving oocyte quality probably because of a less severe NEB (Walters et al., 2002b). In contrast with this study in which 120 dairy cows were subjected to OPU only once during a 120 day period after calving, Kendrick at al. (1999) and Gwazdauskas et al. (2000) reported that oocyte quality is the best around day 30 pp and decreases steadily to day 100 pp. However in the latter study, the same cows were punctured twice weekly, a procedure that disturbs follicular turnover and probably alters oocyte quality (Boni et al., 1996). Dairy cows that have been overfed during the dry period to induce

fatty liver and thus a more profound NEB shortly after parturition, produced at 80-120 days pp oocytes with poorer developmental competence in vitro compared to oocytes from control cows (Kruip et al., 1995).

Conclusively, it can be said that thanks to the OPU technique it was possible to demonstrate a reduced oocyte quality in high-yielding dairy cows. This observation may be partly responsible for the reduced fertility in general and more specifically for the low conception rate and higher prevalence of early embryonic mortality. Poor oocyte quality and subsequent disappointing embryonic development may reflect a compromised follicular development and intrafollicular environment directly affecting the maturing oocyte. Also the environment of the follicular fluid (FF), embedding the growing and maturing oocyte, can be perfectly studied by means of the OPU technique.

OPU TECHNIQUE AS AN OPTIMAL APPROACH TO INVESTIGATE THE INTRAFOLLICULAR ENVIRONMENT

In vivo, the FF embeds the granulosa cells and supports the oocyte to undergo the fine tuned process of growth, pre- and final maturation. Intrafollicular conditions are determinant for oocyte quality. It is generally accepted that maternal mRNA and protein molecules are synthesized and accumulated during oocyte growth and maturation (Lonergan, 2003a, Vassena et al. 2003; van den Hurk and Zhao, 2005). The latter is crucial to guarantee the early embryo development prior to embryonic genome activation which occurs at the 16 cell stage in cattle. It has been shown that maturation environment can influence the abundance of several developmentally important gene transcripts in bovine oocytes (Lonergan, 2003b; Watson et al., 2000).

Transposing this knowledge on the specific situation of high-yielding dairy cows, it can be assumed that the oocyte is vulnerable to possible adverse changes in its follicular environment. Britt (1992) hypothesised that the developmental competence of the oocyte and the steroigenic capacity of the follicle in high-yielding dairy cows is determined during the long period (up to 80 days) of follicular growth prior to ovulation. Thus, primordial follicles exposed to adverse conditions associated NEB early pp, are less capable of producing adequate amounts of estrogen or progesterone (after ovulation) (Britt, 1992; Roth et al., 2001). The more, these follicles are doomed to contain an inferior oocyte with genetic abnormalities that will be ovulated around 60-80 days pp. Besides the NEB, also the high energy and protein content in modern dairy cow rations have been suggested to alter the intrafollicular environment, which may affect oocyte quality (Boland et al., 2001; McEvoy et al., 2001; Kenny et al., 2002). Hampered oocyte quality will result in lower conception rates, higher early embryonic mortality and thus prolonged calving to conception intervals. Several mechanisms are suggested to be directly and indirectly harmful for the growing and developing oocyte.

Much attention has already been paid to the effect of NEB on endocrine changes in serum and in the follicle, affecting follicular growth and health (Gong, 2002; Lucy, 2003; Sutton et al., 2003; Webb et al., 2004). Besides the interactions between follicular growth and oocyte quality, it is generally suggested that any endocrine disturbance in the intrafollicular environment is likely to disturb oocyte growth and maturation (Reis et al., 2002). However, only few studies suggested possible influences of

the NEB associated concentrations of glucose, BHB or NEFA on the oocyte quality. The more, little is known about the implications of these pp biochemical serum changes on the composition of the FF. Edwards (1974) suggested already that agents capable of interfering with the oocyte health could be introduced in the preovulatory follicle and could also exert their effects after ovulation, since there are relatively large quantities of FF between the cumulus cells. The follicular environment in high-yielding dairy cows has been studied extensively in our lab. In this way we were able to investigate whether al these metabolic changes in serum, caused by the NEB and high energy and protein diets in high-yielding dairy cows, are reflected in the FF. The OPU protocol we used has been described elsewhere (Leroy et al., 2004b). Briefly, 9 healthy and multiparous high-yielding dairy cows were used to collect blood and FF samples. On day 11 pp all animals showed normal uterine involution and follicular growth on one or both ovaries upon ultrasound examination. On day 14, 20, 26, 33, 40 and 46 pp (experimental sessions), dominant follicles with a diameter greater than 0.8 cm were subjected to ultrasound guided transvaginal aspiration as described by Bols et al. (1995). Briefly, the rectum was emptied and the perineum and external genitalia were cleansed carefully. Cows received epidural anaesthesia (5 cc Procain HCl 4% with adrenalin, Eurovet N.V., Heusden-Zolder, Belgium) to prevent them from straining. An OPU device, equipped with a 5.0 MHz mechanical multi angle probe transducer (Esaote / Pie Medical NV, Maastricht, The Netherlands) and a needle guidance system (Pie Medical) was inserted vaginally and both ovaries were visualized through rectal manipulation. Before aspiration, the number of different sized follicles (< 4 mm, 4-8 mm, and > 8 mm) was recorded per ovary. Subsequently, follicles were punctured and the FF was aspirated by a second operator, following positioning along the biopsy line. Hereto, the needle (TERUMO NEOLUS 21GX2” 0.8X50, Leuven, Belgium) was attached by means of a stainless steal connector to an extra thin silicon tube (inner diameter: 0.034”; Silclear TM Tubing, Multi Purpose Medical Grade Silicone Tubing, Degania Silicone/Israel) and a 5 ml syringe (Plastipak™, Madrid, Spain) was used to aspirate the FF from the punctured follicle. The largest and the second largest follicle (if present) with a diameter greater than 8 mm were aspirated. Attention was paid to prevent blood contamination. Follicular fluid samples with obvious blood contamination were omitted from further processing.

To identify possible atresia of the punctured follicles, a progesterone (P4) and estradiol-17ß (E) analysis was carried out on each FF sample (RIA). Follicular fluid with a ratio E/P4 < 1 was considered to originate from an obviously atretic follicle and was omitted for biochemical analysis (Wise, 1987; Badinga et al., 1992; Landau et al., 2000).

In each sample, the concentrations of glucose, beta-hydroxybutyrate (β-OHB), urea, total protein (TP), triglycerides (TG), non-esterified fatty acids (NEFA) and total cholesterol (TC) were measured using wet chemistry techniques on two clinical chemistry automated analysers (Modular P and Hitachi 911, Roche Diagnostics).

Some of the results are presented below (Leroy et al., 2004b).

Based on the profile of glucose, we concluded that the FF glucose concentrations are very well correlated with serum levels and that they are consistently higher (10%) than in serum. This finding strongly suggests that pp changes in glycaemia are well reflected in the FF of dominant follicles but that the oocyte is more or less ‘protected’ from extremely low glucose concentrations.

Both serum glucose and β-OHB as well as FF glucose and β-OHB were negatively correlated (*r* = -0.56 and -0.83, respectively). The average serum β-OHB concentration peaked at 33 days pp (1.62 mM). This concentration is associated with signs of subclinical ketosis (Busato et al., 2002). The β-OHB concentrations in serum and in the FF of the punctured follicle were similar and both slopes of profile were exactly the same. Based on the good correlation between serum and FF concentrations throughout the experimental period, it can be said that elevated β-OHB levels in the serum (ketonemia) will appear in the FF as well. These findings are consistent with earlier results (Leroy et al., 2004a).

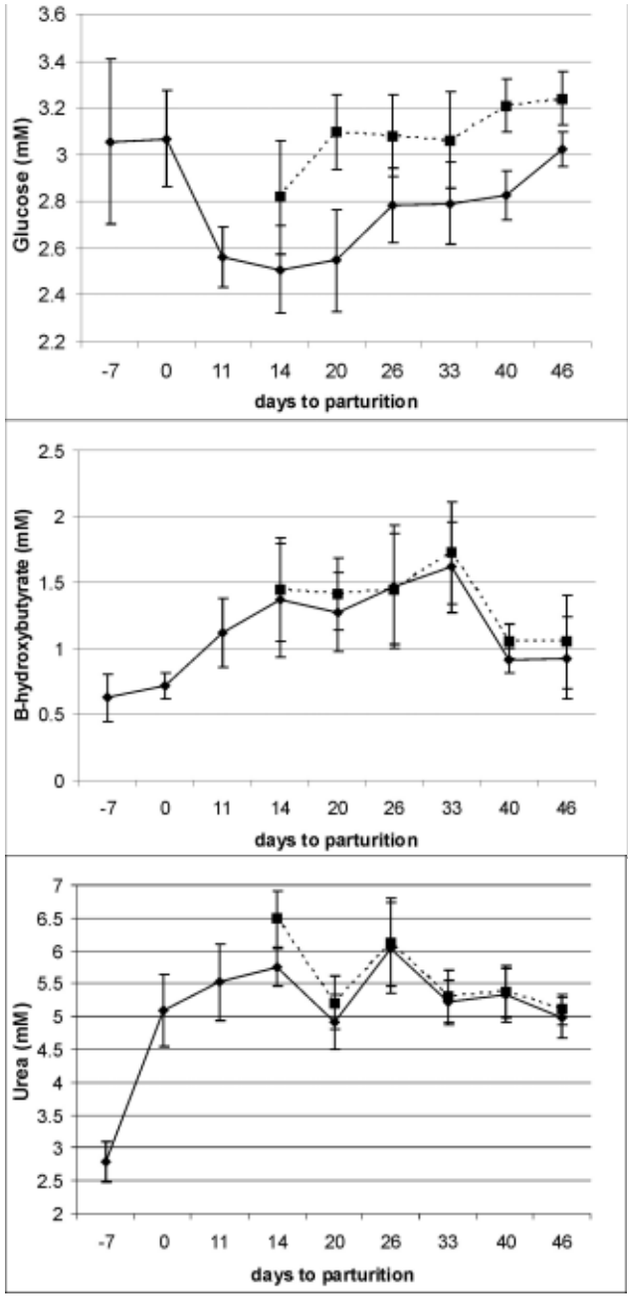

Figure 1. Average (± SEM) glucose (A), ß-hydroxybutyrate (B) and urea (C) concentrations (mM) in serum (black line) and follicular fluid (dotted line) of 9 high-yielding dairy cows during the experimental period.

Urea concentrations showed an important increase during the first week after parturition and remained stable in the weeks thereafter. As Leroy et al. (2004a) and Collins et al. (1997) have already shown in respectively cows and mares post mortem, we also found a very high correlation between FF and serum urea concentrations. Elevated serum urea levels are reflected in the FF and hence, may influence oocyte quality.

During a period of NEB, lipolysis causes an increase of NEFA concentrations in serum during the first weeks pp followed by a steady decrease thereafter. The repeated measurement analysis of the data revealed that there is a parallel decrease in the FF. It has been shown already that acute fasting results in a NEFA rise both in serum and in FF (Comin et al., 2002; Jorritsma et al., 2003). In our study however, the FF concentrations stayed consistently lower than the levels in serum. Furthermore, there was a much higher variation in NEFA concentrations between animals in serum compared to FF (an average coefficient of variation of 58% and 30%, respectively). Both findings suggest that there may be a mechanism to protect the oocyte and the granulosa cells from possible toxic effects of high NEFA concentrations, which are shown to occur in vitro (Mu et al. 2001; Vanholder et al., 2003). In consequent research we specifically focused on the NEFA concentration and composition in serum and in FF of high-yielding dairy cows during and shortly after the period of NEB. As shown in figure 4 it was confirmed that the NEFA concentrations in FF also rise due to NEB but remain significantly lower than in serum. The more, also the NEFA composition differs between serum and FF (Leroy et al., 2005).

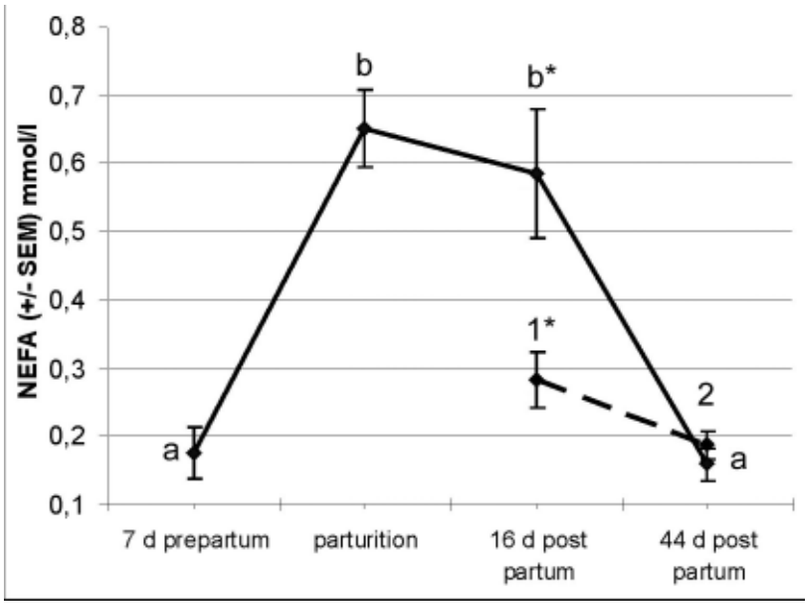

Figure 2. Mean absolute non-esterified fatty acid (NEFA) concentrations (± SEM) in serum (black line) and in follicular fluid (dotted line) at different time points to parturition. Serum NEFA concentrations, marked with a, b, differ significantly between different time points. Follicular fluid NEFA concentrations marked with 1, 2 differ significantly between different time points. Non-esterified fatty acid concentrations at one time point marked with *, differ significantly between serum and follicular fluid ($P < 0.05$).

In conclusion, using the adapted OPU technique it was shown for the first time that the biochemical composition of the FF is well correlated with the composition of serum and that the typical biochemical changes during the first weeks pp are well reflected in the FF of the dominant follicle. This finding may be an important factor in the pathogenesis of the subfertility in high-yielding dairy cattle, by affecting the quality of both the oocyte and the granulosa cells.

VALUABLE KNOWLEDGE FOR FURTHER IN VITRO STUDIES

The second important step in this research path is now to investigate whether these NEB associated FF concentrations of for example urea, β-OHB, glucose or NEFA are directly toxic for the oocyte or granulosa cells. Since it is very difficult to investigate this hypothesis in living animals, we developed in vitro maturation models to investigate the toxicity of high NEFA or high β-OHB concentrations (Leroy et al., 2005). Also the effect of hypoglycaemic conditions on the oocyte maturation and subsequent in vitro developmental competence was tested.

Kruip and Kemp (1999) suggested already possible direct toxic effects of high NEFA levels at the level of the ovary. Jorritsma et al. (2004) demonstrated toxic effects of supraphysiological concentrations of albumin bound oleic acid on the oocyte maturation in vitro. Homa and Brown (1992) described similar effects of linoleic acid. The toxic effects of elevated NEFA concentrations have also been documented in bovine (Vanholder et al., 2005) and human granulosa cells (Mu et al., 2001), in Leydig cells (Lu et al., 2003) and pancreatic ß-cells (Maedler et al., 2001). In our in vitro study, we tested the effect of NEB associated concentrations of the three most important fatty acids present in FF: oleic acid (OA, C18:1), palmitic acid (PA, C16:0) and stearic acid (SA, C18:0). Addition of PA or SA during oocyte maturation had negative effects on maturation, fertilization and cleavage rate and blastocyst yield. More (late) apoptotic cumulus cells were observed in cumulus oocyte complexes matured in presence of SA or PA. OA had no effect at all.

Another important feature of NEB are the very low glucose and elevated β-OHB concentrations both in serum and in FF (Leroy et al., 2004b). Preliminary results indicate that especially the hypoglycaemic conditions rather than the high ketone body concentrations during in vitro maturation are detrimental for the oocyte's developmental capacity (Leroy et al., unpublished).

Due to high-protein diets, urea rises both in serum and in FF. It has been shown that high urea levels can be harmful for oocyte quality. De Wit et al. (2001) reported a retarded nuclear maturation and reduced fertilization and cleavage rates in bovine oocytes matured in the presence of 6 mM urea probably through inhibition of the polymerization of tubulin into microtubules. Similar toxic effects on oocyte maturation have been documented by Ocon and Hansen (2003).

The data of these studies suggest that toxic effects of elevated FF NEFA or urea concentrations or very low glucose concentrations on oocyte quality may be one of the factors through which NEB exerts its negative effects on fertility in high-yielding dairy cows. These data may be an important step in unravelling the pathogenesis of the reduced fertility in high- yielding dairy cows.

CONCLUSIONS

Thanks to the OPU technique it was possible to study the oocyte quality and the intrafollicular environment in high-yielding dairy cows. All the typical biochemical changes that feature the period of NEB in high-yielding dairy cows can have their impact on the FF composition. Oocytes do seem to be vulnerable to some of these metabolic alterations leading to a hampered developmental competence. This could partly explain the world wide reported disappointing conception rates and higher prevalence of early embryonic mortality in high-yielding dairy cows.

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NEW METHODS ON EQUINE COOLED AND FROZEN SEMEN BIOTECHNOLOGY

Papa, F.O.; Melo, C.M.; Dell'Aqua Jr., J.A.; Macedo, L.P.; Carvalho, A.G.;
Alvarenga, M.A.; Medeiros, A.S.L.

Department of Animal Reproduction and Veterinary Radiology
UNESP, Botucatu S.P., Brazil papa@fmvz.unesp.br

INTRODUCTION

The development of techniques involving storage and preservation is one of the most important steps for the improvement of semen technology. It allows the use of animals with high genetic potential, which are either temporarily or permanently unable of reproductive activity. In addition, those techniques can favor semen shipment to long distances, and are the best biologic insurance for genetically superior stallions. Difficulties to achieve a standard method for equine frozen semen, makes cooled shipped semen the most suitable technique currently used in Brazil. Some of the limiting factors involving the use of frozen semen are: sophisticated equipment costs, necessity of specialized and experienced technician, transportation of supplies for freezing, and adequate facilities for semen processing on farm. The problems found during this process can be overcome with a new technique, which consists of: semen collection, dilution, centrifugation if necessary and pre-cooling using an adequate shipping container. The procedures mentioned above can be performed at the farm before semen shipment to either a specialized laboratory or to a reproductive center. Those facilities provide a safer environment for cryopreservation improving and maintaining the fertility rates obtained when using equine frozen semen.

COOLED SHIPPED SEMEN

Brazil has been considered the second country in the word regarding the use of equine cooled semen. One of the numerous advantages presented by this technology is the possibility of semen collection and processing on farm followed by shipment of cooled semen to different locations for mare insemination. This wider used technology has eliminated expenses and stress associated with transportation of mares to stallion for breeding. In addition, it has improved safety in many health aspects, as the prevention of diseases potentially transmitted to mares on arrival at breeding facilities. The increased use of cooled shipped semen, added to the position assumed by some breed registries, which are now presenting full compliance with the use of frozen semen has motivated numerous investigations in this research area at the Animal Reproduction Section, FMVZ- Unesp, Botucatu.

Experiment I: Effect of two antibiotics on viability and fertility of equine semen cooled to 5 °C (Macedo et al, 2005).

Fifteen ejaculates from five stallions were diluted to $50x10^6$ spermatozoa/mL in a skim milk/ glucose extender (Kenney, et al, 1975) with the addition of either Gentamicin sulfate or Amikacin sulfate, both at 0,2 mg/mL. Samples were evaluated every 24 hours for total and progressive motility by CASA (Computer Assisted Sperm Analysis), and the number of sperm cells moving rapidly (RAP)

was determined. Plasmatic membrane integrity was verified using fluorescent probes (Harrison and Vickers, 1990) and a fertility trail was conducted using two cycles from each of 12 mares. Those mares were inseminated at 36 hours after hCG administration using cooled semen previously held at 5° C for 72 hours, and a pregnancy diagnostic was performed at 15 days post-ovulation by ultrasonography. Sperm parameters and fertility rates are respectively described on Figures 1 and 2. In conclusion, the use of those antibiotics was efficient for bacterial growth control, and the sperm parameters demonstrated that Gentamicin had a detrimental effect when compared to Amikacin ($p < 0.05$). Pregnancy rates did not differ ($p > 0.05$) with the use of either Gentamicin (4 of 12, 33.3%) or Amikacin (8 of 12, 66.7%), which can possibly be attributed to the numbers of A.I performed by this study.

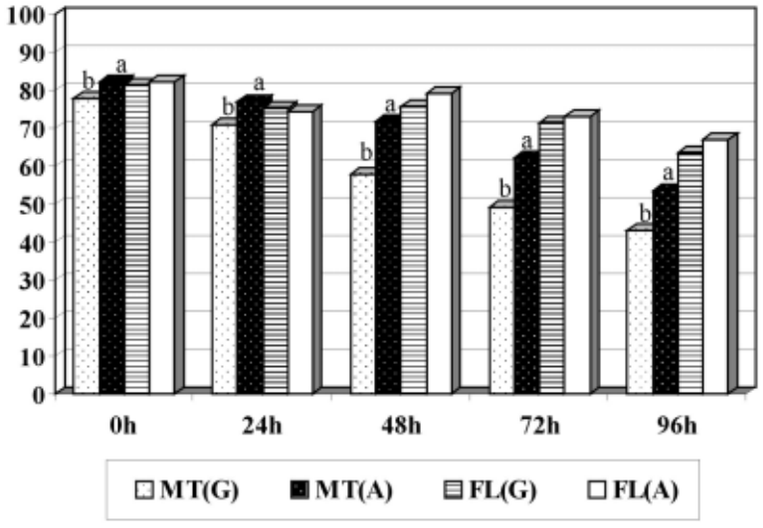

Figure 1. Total motility (TM) and plasmatic membrane integrity (FL) (mean ±SD) of equine semen cooled to 5°C on different moments and antibiotics Gentamicin (G) and Amikacin (A). Means with the same letter subscripts are different ($p<0.05$).

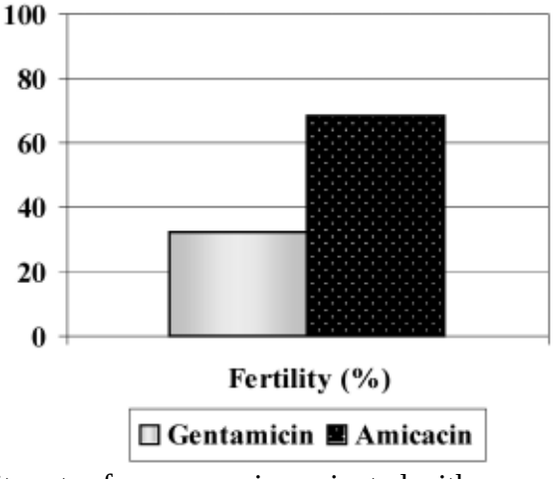

Figure 2. Fertility rates from mares inseminated with semen cooled to 5°C.

Experiment II: Effect of different extenders on sperm parameters of equine cooled semen (Carvalho *et al.*, 2005).

Twenty ejaculates from four stallions were divided into four aliquots, and diluted 1:1 (v:v) in four different extenders: E1 = Kenney, E2 = glycine+egg-yolk+taurine, E3 = glycine+skim-milk, E4 = glycine+skim-milk+taurine, which were then centrifuged at 600x g for 10 min. The sperm pellets were

resuspended in the respective extenders and cooled inside an equitainer™ for 24 hours. After this period, the samples were transferred to an isothermal box containing water with the same temperature reached inside the Equitainer™. This cooling device with samples was then placed inside a refrigerator at 5°C and held for 96 hours at this temperature. The samples were evaluated every 24 hours by CASA and membrane integrity was determined using fluorescent probes. Ninety one inseminations were performed at a fertility trial with the respective number of artificial inseminations (A.I.) per group, extender and cooling period: group 1 (16 A.I., E1/48 hours). Fifteen A.I. were performed in the remaining groups with respective extenders and cooling periods: (E2/48h, E3/48 h, E4/72 h and E4/96 h). Pregnancy diagnostic was performed at 16 days after ovulation with the laboratory and fertility data described respectively on Figure 3, 4 and 5.

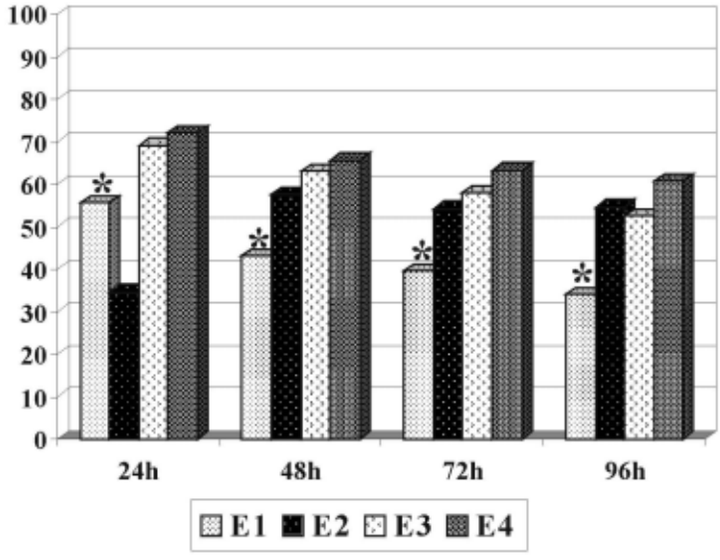

Figure 3. Means of total motility using different extenders at the following cooling periods: 24h, 48h, 72h and 96h. *Significance was set at $P < 0.05$.

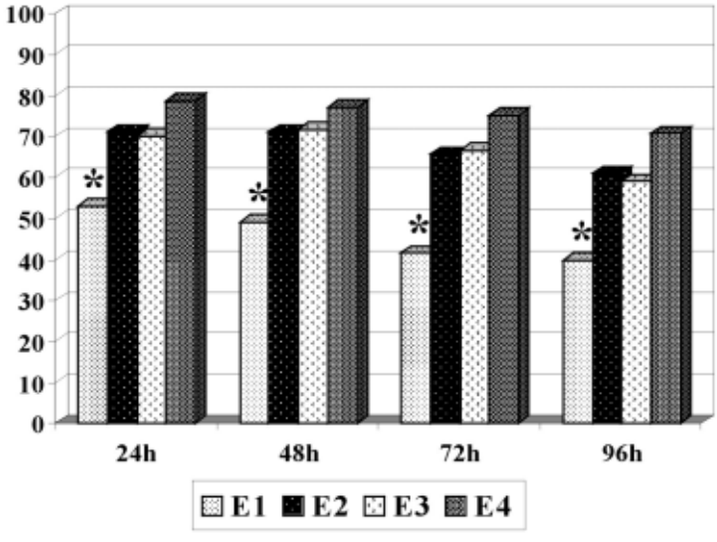

Figure 4. Membrane integrity Means using different extenders at the following cooling periods: 24h, 48h, 72h and 96h. *Significance was set at $P < 0.05$

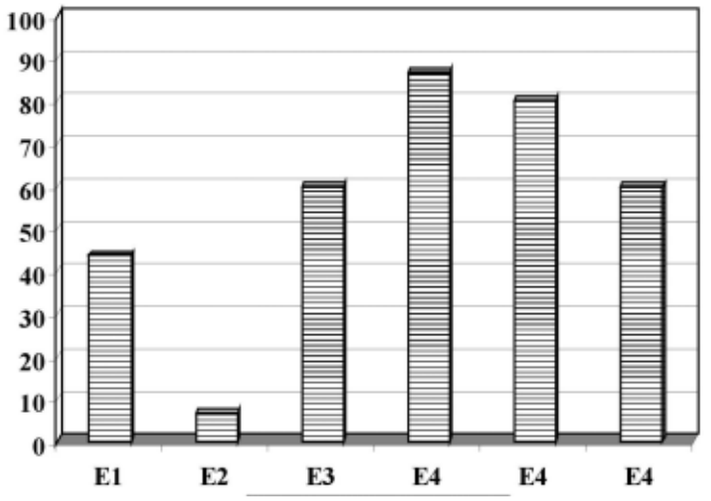

Figure 5. Pregnancy rates for extenders at different cooling periods.

There was no significant difference on spermatic parameters, except for E1 within the cooling periods utilized. The association glycine+skim-milk+taurine represented by E4 maintained the sperm parameters and fertility rates when cooled semen was stored for 48, 72 and 96 hours.

COOLED – THEN FROZEN EQUINE SEMEN

Experiment I: Effect of 24 hours cooling period before freezing on equine semen fertility (Melo *et al*, 2005).

Thirteen ejaculates from different stallions were evaluated by CASA and membrane integrity determined using fluorescent probes. The semen was diluted in Botu-Semen™ and divided into two samples, which were either frozen (Papa et al, 2002) as a control group or diluted to $50x10^6$ sperm/mL, cooled into an Equitainer™ and held for 24 hours before freezing, as a treatment group. The mares were inseminated at the tip of the uterine horn pre and post ovulation with 350×10^6 viable sperm using a flexible pipette. The laboratory data and the fertility trial results are shown on Table 1.

Table 1. Percentage means (± S.D) Total motile (TM), Progressive motile (PM), Membrane integrity (FL), Sperm and Fertility rates (FERT) between stallions using either conventionally frozen semen or cooled–then frozen equine semen

Stallion	Treatment	TM (%)	PM (%)	FL (%)	FERT (%)
A	Conventional	71.00±5.33	40.67±6.8	46.67±8.57	72.7
A	Cooled/frozen	73.67±3.88	37.00±8.25	46.5±6.09	83.3
B	Conventional	73.33±6.22	34.67±2.58	53.83±6.08	40.0
B	Cooled/frozen	68.67±10.11	22.67±3.27	54.17±8.08	50.0

Sperm parameters and fertility rates were not affected when semen was cooled for 24 hours before freezing. In conclusion, similarly to cooled semen technology, it is now possible to collect the semen on farm followed by 24 hours of cooling into an Equitainer™ and then ship the semen to either a reproductive center or to a specialized laboratory for cryopreservation.

FROZEN SEMEN

Experiment I: Use of MP50 extender for equine semen cryopreservation (Papa *et al*, 2002).

Seventy-eight ejaculates from 5 stallions were evaluated by CASA and membrane integrity was determined using fluorescent probes. The semen was centrifuged, the pellets were resuspended in MP50 to 200 x 10^6 sperm/ml, placed into 0.5 mL straws and submitted for freezing. A fertility trial was conducted using 12 mares, which were inseminated with 800 x 10^6 total sperm at the uterine body within a maximum of 6 hours post ovulation. The gestation diagnostic was performed 12 days after ovulation and resulted in a pregnancy rate of 75% (9/12). The MP50 extender, commercially known as Botu-Crio™ was able to maintain semen viability and fertility after cryopreservation. This extender has contributed for the wider use of equine frozen semen and it has been efficiently used for frozen semen from different stallions. Genetic variability and biological insurance for animals with high genetic value can be achieved by the use of Botu-Crio™.

Experiment II: The influence of sperm number and stabilization period on equine frozen semen-using Botu-Crio™ (Blanes *et al*, 2005).

Fifteen ejaculates from 5 different stallions were diluted in Botu-Semen™, centrifuged, and the pellets were resuspended using Botu-Crio™ extender. Semen was then placed into 0.5mL straws using the following sperm concentrations: (C1) 200 x 10^6, (C2) 150 x 10^6, (C3) 100 x 10^6 and (C4) 50 x 10^6. Part of those straws were submitted to cooling at 5ºC for 20 minutes and the other straws were maintained at this temperature for 60 minutes until being emerged into liquid nitrogen (N_2) as shown on Table 5. The samples were thawed in a water- bath at 46ºC for 20" (Dell'aqua et al, 2001), submitted to CASA and membrane integrity was determined using fluorescent probes. There was no statistical differences on membrane integrity, total and progressive motility values when different sperm concentrations were used. The samples that were stabilized at 5° C for 20 minutes presented superior TM values when compared to samples that were submitted for 60 minutes using Botu-Crio™ as shown on Figure 6.

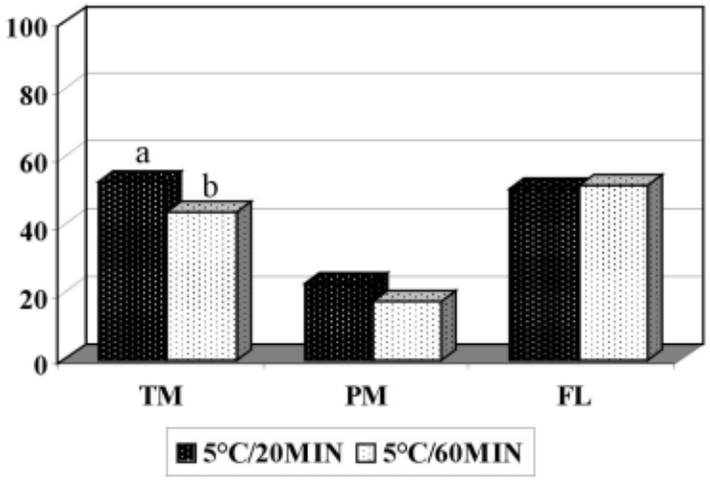

Figure 6. Membrane integrity (FL), total motility (TM) and progressive motility (PM) medium values in relationship to stabilization time at 5º C. Means with the same letter subscript did not differ (p> 0.05).

Experiment III: Equine frozen semen using different amides (Medeiros et al, 2002).

Two ejaculates of 20 stallions from different breeds presenting motility higher than 60% were frozen into 0.5 mL straws using INRA 82 with different cryoprotectants: Dimethyl acetamide at 1, 3 and 5%, Methyl formamide at 5% and Glycerol at 5%. After thawing the samples were submitted to CASA and membrane integrity was determined using fluorescent probes, Figure 7. Thirty inseminations were performed with frozen semen, with either Dimethyl acetamide or Glycerol as cryoprotectants, and fertility rates of 40% (6/15) and 0% (0/15) were respectively found. The present experiment shows that the amides in general are able to better protect the spermatozoa during the freezing and thawing process. In conclusion, this cryoprotectant is capable of maintaining acceptable fertility rates if compared to glycerol when used in stallions considered poor freezers.

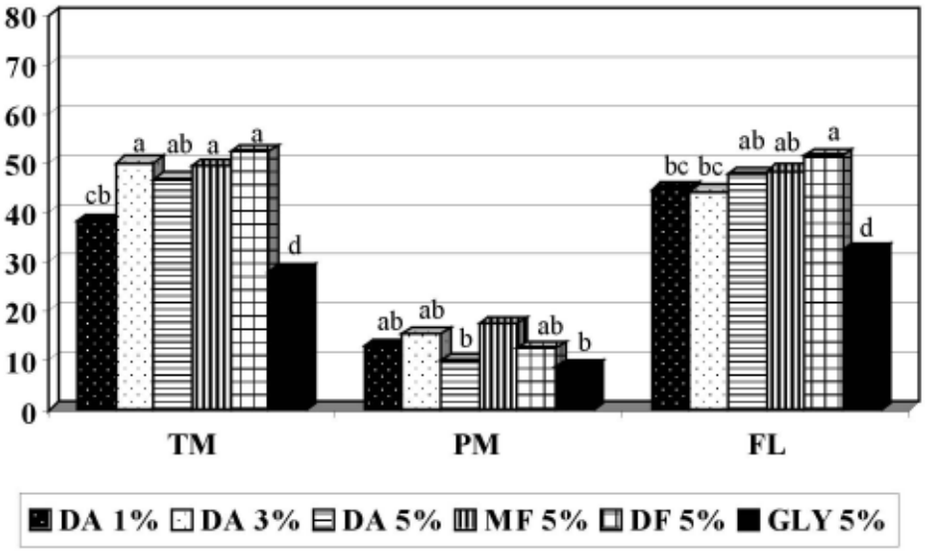

Figure 7. Percentage means Total Motile (TM), Progressive Motile (PM), and Membrane Integrity (MI) sperm, when stallion spermatozoa were cryopreserved using different cryoprotectants.

Experiment IV: Effect of sperm concentration and centrifugation on equine frozen semen (Dell'Aqua Jr. & Papa, 2001).

Twelve ejaculates from different stallions were centrifuged to remove seminal plasma. Nine different protocols evaluated the influence of time and force of centrifugation on spermatozoa recovers and their effects on sperm parameters. The ejaculates were diluted at 1:1 (semen: extender) using a solution with 50% skim-milk + 50% Ringer'S Lactate and then centrifuged using different protocols: P1 (600 x g/3min); P2 (800 x g/3min); P3 (1000 x g/3min); P4 (600 x g/5min); P5 (800 x g/5min); P6 (1000 x g/5min); P7 (600 x g/10min); P8 (800 x g/10min); P9 (1000 x g/10min). Sperm parameters are presented on Figure 8.

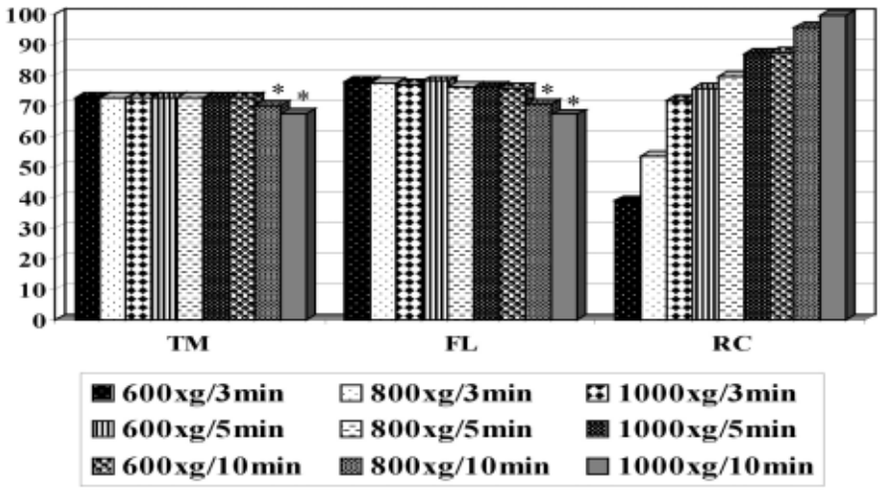

Figure 8. Percentage Means of Total Motility (TM), Membrane Integrity (FL), Sperm Recovery (RC) using different protocols of centrifugation. *Significance was set at $P < 0.05$.

The results demonstrate that semen centrifuged at 600xg for 10min, recovered 87% of initial concentration and had no detrimental effect on either sperm motility or sperm membrane integrity.

Experiment V: Effect of packing system and warming rate on sperm parameters of equine frozen semen (Dell'Aqua Jr. & Papa, 2001).

Twelve ejaculates from different stallions were used to verify the influence of packing system and warming rates on sperm parameters of equine frozen semen. Three packing systems were used: 0.25mL straw, 0.5mL straw and 4mL macrotube at different warming conditions: 38°C, 46°C and 65°C. The thawing period used corresponded to the interval necessary to reach a temperature of 35ºC inside each packing system. The thawing conditions and packing systems were respectively: 38ºC/30", 46°C/15" and 65°C/7" for 0.25mL straws; 38°C/40", 46°C/20" and 65°C/10" for 0.5mL straws and 38°C/90", 46°C/50" and 65°C/30" for 4mL macrotubes. Sperm parameters were evaluated by CASA and fluorescent probes (Harrison e Vickers, 1990) Figure 9.

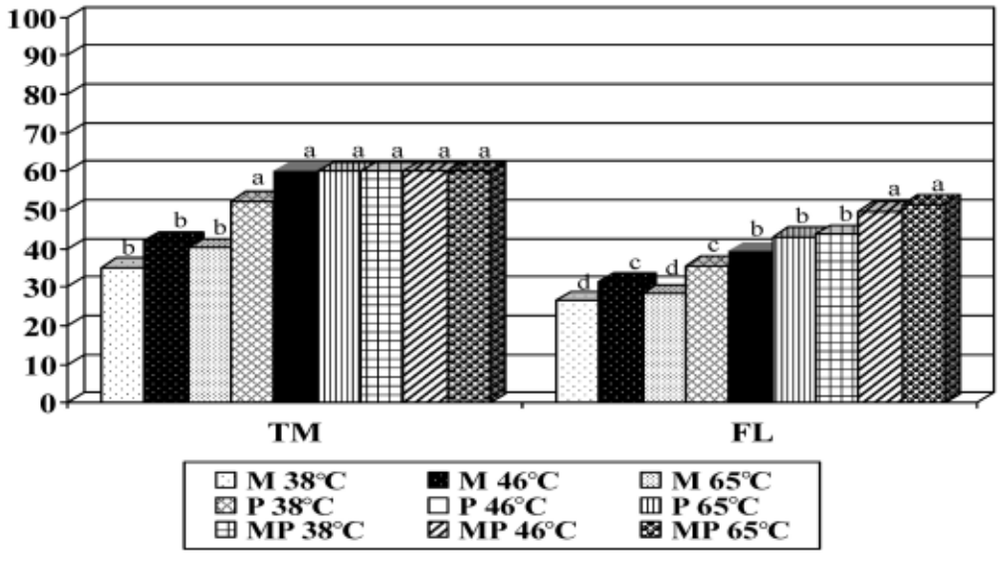

Figure 9. Percentage Means of Total Motility (TM), Membrane Integrity (FL), Sperm Recovery (RC) using different packing systems and warming rates. *Significance was set at $P < 0.05$.

The results suggest that there was no difference between the packing systems used (0.25mL, 0.5mL straws and 4mL macrotubes). Sperm parameters were improved when high thawing temperatures were used except for 4mL macrotubes, which maintained membrane integrity when submitted to intermediate thawing temperatures.

Experiment VI: Effect of insemination site and dose on fertility using equine frozen semen (Dell'Aqua Jr. & Papa, 2001).

Seventy mares were divided into 6 groups to verify the effect of insemination site and dose on equine frozen semen. A fertility trial was carried out using frozen semen from one stallion with well-known fertility. Semen was frozen in 0.25mL and 0.5mL straws. Inseminations were performed twice, being before and after ovulation (within 6 hours of ovulation): G1 = $50x10^6$ sptz before ovulation with one 0,25 mL straw at uterine body; G2 = $50x10^6$ sptz before ovulation with one 0.25 mL straw towards the tip of the uterine horn; G3 = $150x10^6$ sptz with one 0,5 mL straw before and after ovulation at uterine body; G4 = $150x10^6$ sptz with one 0,5 mL straw before and after ovulation towards the tip of the horn; G5 = $800x10^6$sptz with eight 0,5 mL straws after ovulation at the uterine body; G6 = $400x10^6$ sptz with eight (4/4) 0,5 mL straws before and after ovulation at the uterine body; G7 = $50x10^6$ sptz with one 0,25 mL straw, before and after ovulation at the uterine body. Fertility rates are presented on Figure 10.

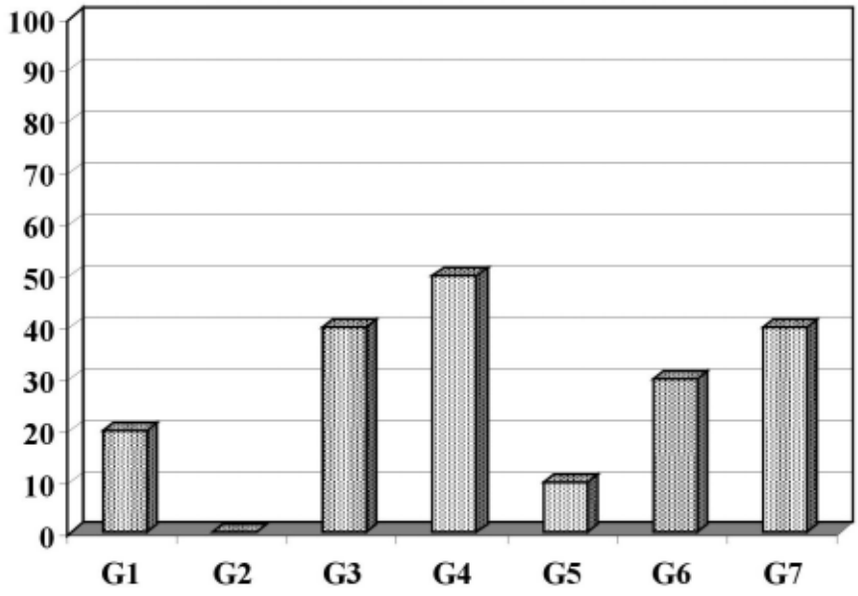

Figure 10. Percentage Means from different insemination protocols.

In conclusion, this fertility trial suggests that low dose insemination can have similar fertility when compared with conventional protocols. In addition, inseminations performed before and after ovulation revealed better results than a single insemination.

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Gusmão A.L. & Andrade Moura J.C. 2005. **Sheep and goat embryo transfer.** *Acta Scientiae Veterinariae*. 33 (Suppl 1): 29-32.

SHEEP AND GOAT EMBRYO TRANSFER

Gusmão, A.L.; Andrade Moura, J.C.

Escola de Medicina Veterinária da UFBA, Av.Ademar de Barros,500- Ondina, 40170110-Salvador – Bahia, gusmao@ufba.br

1- Introduction

Sheep and goat heard production is a major livestock activity worldwide, specialy in countries undergoing development or underdeveloped. This fact is not attributed to livestock option, government support or due to market tendency, but to the incredible capacity presented by those species in adaptation to different conditions, in respect to environment and severe whether all over the world.

Those species exploration has been much more linked to sub-existence activity, without stability in its production chain, lacking development interaction, and without philosophical modernization view. In addition, advanced technologies and financial support for qualified professional education are lacking in this area. Despite of the limitations encountered, there is a growing demand for reproductive technologies, which would lead to increasing productivity and better profitability from herds and production units.

Among those techniques we can emphasize: estrus synchronization, artificial insemination, early pregnancy diagnosis, embryo transfer (ET), oocyte recovery, lab embryo production, and *in vitro* fertilization.

Despite of the fact that the first report on a successfully achieved ET in goats had been published around the 30's (WARWICK et al. 1934), the attempts on amplifying this technology were much later described, by MOORE (1974), TERVIT et al.(1983), CHEMINEAU et al.(1986) and AGRAWAL & GOEL (1991),

2- Sheep and Goat ET Advantages

Among the several advantages presented by ET, the expansion of animal population carrying desirable traits allows the introduction of exotic breeds with high potential of production and maximizes disease control, which reduces and favors costs on importation and exportation of superior genetic material.

3- Sheep and Goat ET Limitations

The main limitation encountered by small ruminant ET has been always related to two factors: costs and surgical methodology applied during embryo collection.

4- Superovulation

The protocols used in our routine work for sheep and goats are cited bellow:

A) GOATS

DAY		TIME		Comments
0			½ CRESTAR placement	Sponge inserted in the Recipients
1				
11		6 am 6 pm	2,5 mL folltropin 2,5 mL folltropin	
12		6 am 6 pm	1,5 mL folltropin 1,5 mL folltropin	
13		6 am 6 pm	1,0 mL folltropin + CIOSIN 0,2 mL 1,0 mL folltropin + CIOSIN 0,2 mL	Sponge removed from the recipient + 300U.I. eCG CRESTAR removal from the donor in the afternoon
14			Artificial insemination or natural matting	
15		6am	Artificial insemination or natural matting	
16				New CRESTAR
20		9 am	Water and food removal	CRESTAR removed + CIOSIN (0,4 mL) later in the afternoon
21		9 am	Embryo collection	

B) SHEEP

DAY	Data	TIME	Procedure	Comments
0		8am	CIDR placement	Sponge inserted Recipients
1				
8				
9		6pm	CIDR changed	
10				
11		6pm	2,4 ml folltropin	
12		6am 6pm	2,4ml folltropin 1,8ml folltropin	
13		6am 6pm	1,8 ml folltropin 1,6ml folltropin	Sponge removed from recipient + 400UI eCG
14		6am	1,2ml folltropin + 200UI eCG 1,0ml (NOVORMON)	**CIDR removed at 6 am**
15		6 am 6pm	Removing food for animals *Laparoscopic insemination*	Insemination at 36 hours after CIDR removal
20		Noon	Remove food and e water	No food for Recipients
21		8 am	Embryo collection	

5- Sheep and goat embryo collection and evaluation

In our particular point of view, studies developed in Brazil from PEREIRA et al. (1991) and PEREIRA et al. (1998) were fundamentally important in making ET, a popular technique for goats, being comparable to the experiments from ELSDEN et al. (1978) in cows.

The main factor that limits the sheep ET use in a large commercial scale has always been the difficult in performing the collection procedure using a trans-cervical approach. Those techniques carried the large disadvantage of equipment high costs, donor genital system adhesions, reduced number of collection performed from a same female; and some times, compromised future reproductive life. Recently, it was possible to overcome this barrier, using a trans-cervical approach for collection in lab routine (SILVA, et al.2001; SILVA et al. 2004). The catheter, Nelaton - Robinson, (Ruesch, Nº Ref. 220500 for goat and Nº Ref. 238500 for sheep), without a balloon, (SUYADI et al. 2000, SILVA et al. 2004) is introduced inside the uterus with the help of a stylet, after the cervical oz being fixed by a Posi. The collection media is infused by a catheter syringe tip, using a volume which varies between 10 and 30 mLs each time. In our routine, normally 250 mLs are infused in each horn. The placement of 200 µg of Misoprostol (Cytotec®) at the cranial vaginal compartment, around 3 to 5 hours before collection helps catheter introduction and placement (SILVA et al. 2004). Recently, SALLES (2002) and GUSMAO et al.(2002) described a methodology that permits embryo collection in goats and sheep, using a cervical approach with a closed flushing system. An alternative for the replacement of a Nelaton – Robinson catheter, having a low cost and easy acquisition, would be the use of a nasal-gastric tube, Nº10 or 12, used in human medicine. However, those tubes only allow a syringe attachment, and they are not strait but too much flexible, making manipulation difficult, and predisposing bending inside the uterus. The flush is retrieved and maintained at an environmental temperature (25 a 30º C), until transfer.

Sheep and goat embryo classification follows the same pattern described in bovine, according to the method reported by LINDNER and WRIGHT(1983). In addition, it follows the international embryo transfer society (IETS) guidelines.

6- Transfer

The transfer is performed by a semi-laparoscopic approach using Ketamine and Xylazine to sedate the animal. After exposure of the uterine horn, epsilateral to ovulation, the embryo is injected using an unopet.

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TWENTY YEARS HISTORY OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (1985-2005)

Mara Iolanda Batistella Rubin

MV. MSc. DMV – Laboratory of Animal Embryology, Veterinary Teaching Hospital Federal University of Santa Maria, 97.105-900 - Santa Maria/RS – Brazil - mararubin@smail.ufsm.br

Summarizing 20 years of history of the Brazilian Embryo Technology Society (SBTE) is a real challenge. Dr. Alvarenga, the president of SBTE, gave this difficult task to me. I spent a great deal of time thinking about, on what would be the most important facts to recount for the young professionals and pay homage to the founders of this society. I will do my best to describe the most remarkable facts and achievements during this period of time.

The SBTE was created to materialize the dreams and expectations of its members. It was created to be the main forum for scientific discussion in embryo transfer (ET). Thanks to Dr. Assis Roberto de Bem, who was the first, yet temporary, president of the society, it was possible to get the early stage of the SBTE organized. Although the SBTE was not technically born until 1985, when the president and the Board of Directors were elected, the true history of embryo transfer begins many years prior to this.

Do you realize how it was for our mentors, around 20 years ago? The flushing material, stressful preparation of the media used to collect embryos, from siliconized glass watches and Petri dishes, surgical transfer of embryos and estrus synchronization with prostaglandin were present in our routines. Everything comes to our minds as a replay of a movie. In contrast, today we have all the disposable material, supplies ready to go, from commercial media to direct transfer, progesterone implants, last generation ultrasound equipment, as well as efficient transport and communication systems, all available to the modern veterinarian.

The first attempt to transfer fresh embryos (Holstein breed) from West Germany was done on April 26, 1977 at the Federal University of Santa Maria (UFSM), in Santa Maria/RS in Southern Brazil, under the supervision of Prof. Dr. Joachim Hahn from the Veterinary School of Hannover [1]. Unfortunately, the embryos were transferred but no pregnancies were obtained at that time Additionally, five donors were superovulated without success.

Exactly one year later, Walter Antonio de Pádua Becker, who was Professor at UNESP-Jaboticabal/SP since 1975, started to work with TE (1978) in Germany. Walter came to Brazil and at the end of May, 1978 he received Prof. Hahn at Guarulhos Airport (SP) with Fleckvieh embryos transported in a thermo bottle. In March, 1979 the first Brazilian's ET calves were born from imported embryos [5,25]. Ariano (03/19/1979), a male Fleckvieh calf became an IA Bull and Odisséia, the female calf, was given to the Brazilian President João Batista Figueiredo. In addition to being a surgeon, Walter was known for his dynamic personality. He envisioned that the Company "Agropecuária Lagoa da Serra" would become one of the most important IA companies for expanding cattle herds, not only in Brazil but worldwide. He built the ET services at the "Lagoa da Serra" [25] where he served as CEO for many years. Walter had Dr. Douglas Brasil Gaetti, Dr. Claudemir de Carvalho and Dr. Carlos Fernando

Marins Rodrigues as his collaborators, which also gave an important contribution during the expansion of the ET techniques in Brazil.

After the first attempt, the pioneer work with collection and transfer of bovine embryos resulting in successful was performed in Brazil at the beginning of 1979 by Dr. Jorge Nicolau Neto's team, from São João da Boa Vista/SP. After graduation at USP in 1976, he moved in January 1977 to the United States where he studied with Dr. Robert Baker and Dr. John Hasler. Jorge organized, in January 1979, the ET program that became headline news in October [18] with the birth of "Eureka" [28], the first of four calves obtained with ET in a Holstein red and white cow at the "Associação São Pedro de Pesquisa Científica" in Sorocaba/SP [25]. On March 14, 1984, when the first Brazilian calf produced from frozen a embryo was born, Nicolau's pioneering was consolidated again [9]. Overall, from 1979 to the present, Dr. Nicolau's team has performed around 90,000 embryo transfers. He was very active in dealing with technical and legal aspects in both the state and the federal arena to organize the trade of embryos.

In 1995, as a direct result of his professional efforts, he accomplished the most important project of his life: he created his own embryo transfer company, the well know Embryonic in São João da Boa Vista/SP, where embryo sexing service is also available. Recently, due to his high professional standards he was invited by the Government of China to coordinate an ET program in that country. The members of SBTE are proud to share the same organization with this exceptional veterinarian and leader. Jorge granted us with his charismatic sense of humor, commitment to excellence and high standards of ethic conduct, one example to be followed.

On May 27, 1979, at the Campo Verde Empreendimentos Rurais Ltd Farm, in Antonio Gonçalves district (Senhor do Bonfim), State of Bahia, Nelore cows were flushed for the first time, in history. Five Nelore calves were born out of seventeen pregnancies [8,14]. Dr. Roberto Mozer de Abreu and Dr. Aurelino Menarim Júnior elaborated the ET program under supervision of Dr. Peter Elsden from Australia and Dr. Larry Nelson from USA. In 1980, these four doctors were able to share the first successful birth of the first five Nelore calves in the world* [25,26].

In the spring of 1979, with the help of Dr. Hahn, the UFSM team and its coordinator, Dr. Carlos Antonio Mondino Silva, with whom I had the privilege to work with as an undergraduate student, introduced the ET technique in the State of Rio Grande do Sul, Southern Brazil, along with Dr. Ricardo Macedo Gregory´s team, from the Federal University of Rio Grande do Sul (UFRGS/RS). José Luiz Rodrigues, a graduate student, was also involved in the ET activities. All of us from the South concluded our doctoral degrees at the School of Veterinary Medicine, in Hannover. We acknowledge our Germany advisers for their support and dedication to our scientific background.

Prof. Hahn brought 15 Fleckvieh embryos [25], and unfortunately, due to intercontinental transportation problems they did not get to their destination on time, arriving 36h after collection. Only 6 transfers (11/8/1979) from these imported embryos from Neustadt a.d. Aisch were performed resulting in one pregnancy. Eighteen donors from three different breeds superovulated days before Prof. Hahn's arrival resulting in 13 pregnancies [22] and the first calf was born on August 5, 1980 [33].

In the state of Sao Paulo, Dr. Roberto Jorge Chebel, from Campinas gave his outstanding contribution through his dynamic and entrepreneurial vision, showing us the practical application of

* Personal information - Aurelino Menarim Junior. June 4, 2005.

ET. Because of his large experience in the subject he became the mentor of several veterinary students and also other professionals during their training; today all are colleagues and members of SBTE. Chebel got his degree in 1972 at the USP-São Paulo/SP. He was an outstanding veterinarian and we all are very proud to follow his mentorship and share friendship. He was very charismatic, friendly and positive in his way to address all the issues during our discussions; he always made our meeting room tremble when he spoke. His colleagues, amazed with his knowledge, always surrounded him.

In March 1985, an International Workshop in ET was held in Brasília/DF organized by a Company called "Stracta" directed by Mr. Pedro Ivan Guimarães Rogêdo. A very competent team of veterinarians such as Dr. Carlos Alberto Zanenga, Dr. Aurenilda da Silva, Dr. Roberto Raulino Lamego and Dr. Aurelino Menarim Júnior were there to support Mr. Pedro. These veterinarians, along with other researchers from the "Centro Nacional de Recursos Genéticos (Cenargen-Embrapa-Brasília/DF)" as well as Dr. Assis Roberto de Bem and Dr. Theodoro Romano Vaske, conducted several scientific projects at the ET Unit.

In the same meeting, Dr. Peter Elsden from Fort-Collins, Colorado/USA and Dr. Rueben J. Mapleloft from the University Saskatchewan/Canada, gave us two brilliant presentations about their work. The expertise of these two outstanding scientists, members of the International Embryo Transfer Society (IETS), gave us the necessary motivation and inspiration to create the Brazilian Society of Embryo Transfer. We are all grateful for their contribution.

Among the speakers, Drs. Assis Robert de Bem, João Soares da Veiga (USP-São Paulo/SP), Francisco Megale (UFMG-Belo Horizonte/MG) and Cláudio Alves Pimentel (UFPel-Pelotas/RS), to name a few, represented our country.

So, on April 25th, 1985 the SBTE was born.

Embryo Transfer Meeting - Stracta Genética e Reprodução Farm
April 26, 1985. Brasília/DF

Below is a list of participants at the first meeting, which had the aim of organizing the SBTE. It was at that meeting that Dr. Assis Roberto de Bem was elected the temporary President of SBTE.

Table 1. List of the founders of SBTE (April, 1985).

Antonio Renato Pacheco	
Assis Roberto de Bem – President	Nilson Antonio Azevedo
Carlos M. Jaume Eggleton	Oziel Buzutti
Cezar Roberto Esper	Paulo Bayard Dias Gonçalves
Evâneo Nogueira Coelho	Paulo Eymard Correa Loureiro
Guenter Kluge	Peter Elsden
Jairo Pereira Neves	Raul Gastão Muciolo
Jorge José Bangel Junior	Roberto Raulino Lamego – Secretary
Leônidas Antonio Chow Castilho	Teodoro Romano Vaske**
Luiz Eustáquio Lopes Pinheiro	Tirzá Portela de Andrade
Mara Iolanda Batistella Rubin	Vicente Otávio Fonseca
Marc Roger Jean Marie Henry	Antonio Carlos Batista**
Marília Viviane de Oliveira-Simões	Carlos Alberto Zanenga**
Nei Queiroz Silva	Francisco Aloisio Fonseca**
Neuci Adalton Vinha	Heraldo Gomes Rangel**
	José Antonio Visintin**

** Voluntary collaborators

On July 18, 1985 during the VI Symposium in Animal Reproduction held in Belo Horizonte/MG, the foundation of SBTE was officially approved by 14 colleagues (Quadro2). The president Dr. Luiz Eustáquio Lopes Pinheiro from the UNESP-Jaboticalbal/SP and the Board of Directors took the lead of the SBTE.

Table 2. List of participants of the Meeting when the SBTE was officially approved (July, 1985).

Luiz Eustáquio Lopes Pinheiro – President	Carlos Alberto Zanenga
Cezar Roberto Esper - Vice-president	Carlos Fernando Marins Rodrigues
Carlos Fernando Marins Rodrigues – Secretary	Carlos Alves Mendes
Irineu Machado Benevides Filho – Treasurer	Claudemir de Carvalho
Assis Roberto de Bem	Evâneo Nogueira Coelho
Aurelino Menarim Júnior	Mara Iolanda Batistella Rubin
Aurenilda Silva	Takeshi Morita

An important mark was the announcement of the birth of the first ET goats, result of Jaume & Bruschi´s work, published by the newspaper “O Estado de Minas” [17]. In the same year, Dr. José Luiz Rodrigues from UFRGS/RS received the Junior Scientist Award given by CNPq, the most important Brazilian financial research agency of the Brazilian Government, in recognition for his work accomplished with Dr. Ricardo Macedo Gregory. Later on, Dr. José Luiz represented our SBTE at several events and at research institutions in Brazil and abroad.

A small number of farmers were aware and had access to the ET technology during the beginning of the 80‘s. In contrast, more than 20 veterinarians were already trained to perform embryo transfer by the time we had the first SBTE scientific meeting in Jaboticabal/SP, in 1986. The founders of the society and our guests had the opportunity to present their scientific papers, including the first work done with

in vitro maturation of bovine oocytes performed by Dr. Joaquim Mansano Garcia and others at UNESP-Jaboticabal/SP.

Still during the mid-80´s the Cenargen-Embrapa, Brasília/DF became a well-known research institution thanks to the pioneer work from Dr. Teodoro R. Vaske and Dr. Assis Roberto de Bem, becoming an important center for training of new researchers in ET technology. Dr. De Bem had produced the first identical bovine twins using embryo-splitting techniques in Brazil. As time went by new teams were formed at USP-São Paulo/SP, UNESP-Botucatu/SP, UFV-Viçosa/MG, along with UNESP-Jaboticabal/SP, UFRGS-Porto Alegre/RS and UFSM-Santa Maria/RS.

During this time, Dr. Leônidas Chow Castilho and others performed several investigations with fresh goat embryos in Minas Gerais [6]. Similar work was also done in Paraná [11] and in Pernambuco [31]. These researchers obtained the first ET goats in Brazil.

In 1987, we got together in Belo Horizonte/MG. The SBTE became more numerous, adopted brothers, like a real family. We spent exhausting hours doing the scientific presentations/discussions. We must consider also the time we spent together having delicious meals at night. We had an election, as determined in the previous meeting, following SBTE regulations. The new board directory had programmed an international congress for 1988.

In Concordia/SC, our friends Dr. Ivo Wentz, Dr. Isabel Regina Scheid, Dr. Ingon Wentz, Dr. Joice Stein Stefani, with Dr. Wolfgang H. Holtz from Göttingen/Alemanha as adviser, began work in swine reproduction, breaking the ground in the area of collection transfer and pregnancies of swine embryos. The result of their work was published in the "Anais do VII Congresso Brasileiro de Reprodução Animal" from Belo Horizonte/MG, in 1987.

In 1988, the new board of directors organized the 3rd scientific meeting in Santa Maria/RS with the presence of Dr. Carlos Munar from Argentina and Dr. Blanca Rusiñol from Uruguay. The high point of the meeting was the work done by Chebel using the ultrasound in the clinical evaluation of recipients and donors. At Cenargen/Embrapa, Dr. Farinasso and his colleagues transferred twenty frozen and ten splitted embryos from Wild Horse of Roraima and Northeastern Donkey. The frozen embryos resulted in the first four Brazilian pregnancies (4/17) in equine. Later on, in November 1988, "Ânima" a female foal, was born [7]. Also Dr. Ricardo Macedo Gregory from the UFRGS/RS, presented his preliminary results of embryo technology applied to ovine species. We should not forget that Dr. Selaive-Villaroel & Mies Filho [23] also from the UFRGS/RS had already obtained positive results with ET in Corriedale ewes in the late 70's.

In 1989, the 4th scientific meeting was held in Porto Alegre/RS. We had the privilege to host Dr. David Whittingham, a pioneer in embryo freezing techniques, who brought to us interesting aspects of embryo cryopreservation of different mammalian species. It is important also to mention the work of embryo cryopreservation done by Dr. Marcos Antonio Lemos de Oliveira team, from UFRPE (PE) in caprine and ET in mares by Dr. João Junqueira Fleury São José do Rio Pardo (SP).

Who can forget at that meeting, the enthusiastic presentation given by our colleague Dr. Fleury? Fleury graduated in Vet Med in 1984 at UNESP/Jaboticabal and, in the following year, he spent about 6 months working with equine embryo transfer under the eyes of Dr. Squires in Fort-Collins, Colorado. In 1986 he performed the first equine ET in our country, and from that moment until December, 2002

he had reached the incredible number of 4,000 ET in mares. Definitively he was the veterinarian who collected/transferred the largest number of equine embryos in the world. His record is recognized in Brazil as well as in other countries. Although debilitated by an illness, Dr. Fleury continued his work sharing his knowledge with others, including his wife Perla Fleury and associates, and by being the mentor of several very capable professionals now working with equine ET in Brazil. His legacy of dedication and enthusiasm will be with us for the years to come.

Our meeting in Porto Alegre went beyond the scientific discussions. We had also the opportunity to enjoy the artistic part of the program.

In 1990, we traveled to Brasilia/DF, where we were introduced to nuclear transfer and cloning by Dr. Lawrence Smith, whose work preceded the production of transgenic animals for scientific and commercial purposes.

In 1991, the scientific meeting was held in Curitiba/PR, under Menarim´s presidence. The scientific board committee invited Dr. Robert K. Baker from "Select Embryos Inc". In his presentation he motivated us to actively participate in exercises evaluating both fresh and frozen embryos through big screen diapositives. The emphasis of the meeting was given to embryo sexing, IVP of bovine embryos and the announcement of the first female calf obtained in 1990 from frozen embryos by the ultra-rapid method in Brazil. We also had intense social activities after the conferences.

When Dr. Enoch Borges de Oliveira Filho (1992), assumed the presidency he expressed his concern regarding the global economy. He believed that the international commerce of embryos should be directed to planning international trade of embryos based on the reality of the market. The presentations were focused in this direction, with bovine fetal sexing and caprine frozen embryos that were imported from France to Ceará [30].

In 1993, Ribeirão Preto/SP held our annual meeting. We celebrated the official recognition of SBTE by the IETS, which was a guarantee that SBTE would participate in all events organized by IETS. In the same year the UNESP-Jaboticabal/SP team coordinated by Dr. Oliveira Filho presented the first three *in vitro* produced *Bos taurus indicus* pregnancies in the world. Three calves were born in October 1993 and pushed our country further ahead in biotechnology. The UNESP-Botucatu/SP, represented by Dr. Cezinande de Meira e Dr. Marco Antônio Alvarenga, reported enthusiastically at SBTE that their first ET-foals out of frozen embryos were born in 1991 [12].

The city of Campinas/SP hosted the 1994 meeting. It was a difficult time to organize a meeting of such proportion being short of funds. However the dedication and efficiency of the team coordinated by our colleagues Sonia Maria Gaspar Paggiaro and Vera Fernanda Hossepian de Lima, collaborated for the SBTE to successful regain it's strength and financial balance. Our Society was strengthened to his original destiny of progress and success.

The team composed of Dr. Eunice Oba (UNESP-Botucatu/SP), Dr. Rubens Paes Arruda (FMVZ-USP-Pirassununga/SP), Dr. José Fernando Garcia, Dr. Pietro Sampaio Baruselli and Dr. José Antonio Visintin (FMVZ-USP-São Paulo/SP) enlightened our meeting with a new focus: the buffalo. Research done in isolation and culture of bovine fetal pre-antral follicles opened new perspectives for investigations (Dr. José Ricardo Figueiredo). In Brasilía/DF, the Cenargen's team was able to share the successful birth of "Vitro", the first pure Nelore breed calf obtained by *in vitro* production [20]***.

*** Personal information - Regivaldo Vieira de Souza. July 1, 2005.

In this same year, our colleague Altino Pires de Almeida Filho gave a brilliant presentation on "Early embryonic mortality in repeat-breeder cows". Altino studied in Israel, where he earned his doctoral degree. In Brazil, he developed his work throughout eight different states. For his work, he was awarded the reputation of being one of the most important ET practitioners in our country.

At the age of 10, leaving behind our childhood and entering adolescence, the SBTE members met again this time in Águas de Lindóia/SP, in September 1995. We were focusing on not only the research activities out in the field, but also on the basic research done in the laboratories such as spermatozoa sexing by centrifugation density gradients. We advanced to the future led by Dr. Vera Hossepian de Lima (UNESP-Jaboticabal/SP) where we were put face to face with a new reality not seen before.

The city of Canela/RS, was the stage of the 1996 meeting. We had the privilege to see the advances of commercial OPU/IVP in the USA by Dr. John F. Hasler and the most recent bovine cryopreservation procedures by Dr. H. Niemann. In that European-like environment, we could see proof of where the research done with follicular growth dynamics in cows had gone. Our honorary and active member of SBTE, Dr. Gabriel Bó, showed us his work on these topics.

The year of 1997 (February) was marked by the birth of Dolly. In Brazil, Cenargen had already started the activities regarding cloning, due in part to the work of Dr. De Bem, who had the help of many colleagues that are still active researchers at Cenargen. Dr. De Bem passed away in September of the same year. He also participated in setting up the "Brazilian Animal Germoplasm Bank". He was an example, both as a mentor and as a researcher, in the University of Lages/SC. His legacy became the scientific foundation for others that came after his time. His questioning about life portrayed his own person. Dr. De Bem always manifested his love for family and friends.

In the same year, Dr. José Luiz Rodrigues was elected as a member of the IETS Governor Board, the first Brazilian to be a member of that organization. In the event in Foz do Iguaçu Falls/PR (1997), Dr. Matthew Wheeler (USA) shed new light over embryonic stem cells and transgenic recent advances. Gottfried Brem, from Bayern-Germany was also one of the speakers that demonstrated the most modern impacts of biotechnology on animal breeding.

During an event organized in Atibaia/SP (1998), the role of the nutritional status of donors and recipients on the reproductive function presented by José Eduardo Portela Santos, broadened our vision on how to correctly manage the cow from the nutritional point of view. From the Marajó Island/PA, a Northern region of Brazil, Otávio Mitio Ohashi's team pointed out important differences in ovarian function between a female *Bos taurus indicu*s and a *Bubalus bulalis*. It was interesting that the female buffalo had smaller follicle populations than the female *Bos taurus indicus*!

Still traveling around the country, we got together this time in Campos do Jordão/SP, in 1999. We discussed the progress and the limitations regarding the *in vitro* production of equine embryos. We very much appreciated the work of Dr. Fernanda da Cruz Landim-Alvarenga who shared her experiences, leading to the opening of a new research area at the UNESP-Botucatu/SP. Dr. Anneliese de Souza Traldi's (USP-Pirassununga/SP) team compared the *in vitro* x *in vivo* criopreservation in caprine and ovine species [27]. From Sobral/CE, Simplício and co-workers we saw the interesting work with caprine pubertal recipients [24]. In that meeting, new perspectives were given with the research in equine

reproduction, particularly with pregnant mares as oocyte donors and a new superovulation protocol presented by the colleagues from UNESP-Botucatu/SP [2] associated with Dr. Ed L. Squires, from Colorado State University. This new protocol could improve the expansion of the equine ET programs. Another interesting topic was presented by Baruselli and co-workers [4] about the follicular dynamic study in buffalos. This meeting was also an opportunity to enjoy the nice weather in the mountains and the excellent food.

In 2000, during the meeting in Rio Quente/GO, we documented the progress of the ovum pick-up for *in vitro production* of bovine embryos that changed from the laboratory setting to the commercial arena. During this time, Dr. Carlos Marins Rodrigues (from Gertec Embriões Ltd.) along with the team of UNESP-Jaboticabal/SP, Dr. Carlos Alberto Zanenga (MS)**,** Dr. André Dayan, Dr. Yeda e Dr. Michele Watanabe (Vitrogen) and the team of Cenargen (Dr. Mauríco Peixer, Dr. Margot A Nunes Dode and Dr. Rodolfo Rumpf) developed a series of new investigations that contributed to progress in this area.

We can not forget the work done by other laboratories such as Cenatte Embriões, In Vitro Brasil, Bio-Reprodução Animal, Klonembryo and Embryovitro, certainly contributed to the improvement of the genetics of the bovine herd in our country. As we know, all these researchers were part of the SBTE family. Considering the recent progress, we felt the need for a change in the name of SBTE, named from that point on as the "Brazilian Society of Embryo Technology". The scientific discussions inside the Hotel room then switched to water games, and we had a great time with this in Goiás.

The turn of **the twenty first century** began with the important scientific achievement named Dolly (1997). The Cenargen-Brasilia/DF, UNESP-Jaboticabal/SP and USP/SP's teams also reached their goal: a successful cloning. In 2001, the birth of Victoria, a healthy female calf, a project coordinated by Dr. Rodolfo Rumpf of Cenargen, marked the beginning of the cloning era in the Brazilian research community.

In the USP-Pirassununga/SP, Dr. Aníbal Moretti's team concluded important research regarding ET in swine [10,29].

Assisted reproduction techniques in caprine were enhanced by Dr. Raimundo Pereira and co-workers (1998), who first developed the non-surgical embryo collection in the goat, in Germany. This technique has been used worldwide. Also working with goat and sheep species, in Brazil, Drs. André Medeiros, Aurino Alves Simplício (CE), Carmen Iara Mazzoni González (PB), Joaquim Correa Andrade, Luiz Roberto D Medeiros, Oswaldo C Gomes Neto, Orlando Procópio and Gustavo Ferrer Carneiro (PE), Jefferson Ferreira da Fonseca (MG), Sérgio Nadal da Luz, Luiz Ernandes Kozicki (PR) [3], Jairo Pereira Neves (RS), Marcos Chalhoub C Lima, Edimilson Almeida Machado and Alberto Gusmão (BA), developed their field work from which non-surgical access in ET was established.

The establishment of new laboratories in different institutions in the late 90´s was a reality. Dr. Alceu Mezzalira from CAV-UDESC (Lages/SC) and Dr. Ligia Margareth C Pegoraro (Embrapa-Pelotas/RS) gave new blood to the work on cryopreservation of bovine oocytes and embryos and also to the OPU/IVP by Dr. Marcelo Marcondes Seneda (UEL-Londrina/PR). The research in *Bos taurus indicus* and *Bubalus bubalis* by Dr. Ciro Moraes Barros and Dr. Jose Luis M de Vasconcelos (UNESP-Botucatu/SP) and Pietro Sampaio Baruselli (USP-Pirassununga/SP) gained recognition for their work. More recently, Dr. José Carlos Deschamps's (UFPel-Pelotas/RS) team has started their ET work in swine.

In January 2002, under the presidency of Dr. Rodolfo Rumpf, the SBTE received the IETS members in Foz do Iguaçu Falls/PR. The team work of the SBTE Board of Directors, IETS president and the Local Organizing Committee coordinated by Dr. José Luiz Rodrigues transformed the event into an important mark in the future meetings organized by IETS. The overall opinion of the participants was that the Symposium had reached an excellent scientific level, had a larger number of participants, excellent facilities and a great variety of food and touristic programs. The event had transformed itself into a scientific forum never seen in the IETS or in the SBTE. Dr. Rumpf's and Dr. Rodrigues's teams worked hard and very well! In the biggest event we had so far, we had the opportunity to meet renowned scientists, authors of very important scientific publications such as Drs. Ian Wilmut, KHS Campbell, WA Ritchie, RJ Mapletoft, S Leibo, J Hasler, P Bredbacka, B Avery, H Callesen, G Vajta, I Lewis, T Nagai, MB Wheeler, E Carnevale, EL Squires, CG Luvoni, BG Brackett, K Hinrichs, SJ Dieleman, H Niemann, E Wolf, G Seidel Jr., M Thibier, B Marquant-Le Guienne, P Humblot and G Bó, to name a few.

To confirm our evolution, in the USP/SP the clone "Marcolino" was born, a male calf originated from differentiated fetal cells [13], and "Penta" was born in July 2002, the first clone obtained from cells of an adult animal resulting from the UNESP-Jaboticabal's work [32]. At the CAV-UDESC-Lages/SC was born "Victra", the first female calf from South America derived from a vitrified embryo obtained by IVF of vitrified immature oocyte, a research from Mezzalira' team.

On September of 2003, the research project on cloning resulted in the birth of "Lenda" at Cenargen/Embrapa, a Holstein female, originated from granulosa cells. At the University of São Paulo/SP, Dr. Maria Angélica Miglino developed studies on IVP and cloned embryo recipient placentas [15]. This year, the SBTE invited Dr. Vilceu Bourdignon, who discussed the progress and future challenges of animal cloning by nuclear transplantation. Beberibe's beaches and tours programs were appreciated as well. On December of 2003, the USP/SP´s team announced that "Bela", a Nelore clone obtained from adult cells, was born. In Porto Alegre/RS, our colleague Berenice de Ávila Rodrigues (UFRGS/RS) developed *in vitro* maturation and fertilization of canine oocytes, a pioneering work in this area, in Brazil.

In February 2004, "Vitoriosa" was born (at Cenargen), a clone of cells taken from the clone "Vitoria". In September, the USP-Pirassununga/SP team coordinated by Dr. Flavio Vieira Meirelles in partnership with Vitrogen, announced the birth of "Independência". These results are the assurance for all the scientific investments for 2005. The birth of the first three commercial clones launched our country further ahead in biotechnology research. This team of researchers has produced a total of 8 cloned animals born up to April 2005 [19].

In March 2005, under Rodolfo Rumpf's coordination, Leonardo Luiz da Silveira earned Cenargen/Embrapa another important achievement: the birth of 2 female foals "Branca and Neve", originated from embryo splitting, thus overcoming a limiting barrier in equine reproduction [16]. This event placed Brazil in the international media, as proof of our high scientific level. It is also important to mention the work of our colleague João Batista Figueiredo da Costa Neto, our friend "Bimbo", who worked with João Junqueira Fleury. Bimbo has transferred the highest number of embryos in Brazil and is also responsible for developing an efficient freezing protocol for equine embryos with more than 55

pregnancies from 1989 to 2005.

Brazil has become the second world leader in equine ET with around 3,500 transfers each year [16]. During the last 5 years some breeders associations changed their regulations allowing the breeders to access the techniques of collection and transfer of embryos. This fact opened new perspectives in terms of research in the equine industry.

Consolidating the methods, of cloning in bovine species, Porã and Potira, the first two calves of Junqueira´s breed were born in March 2005 (Cenargen-Embrapa). This represents the triumph of science and it´s a real proof that this technology can be used to preserve a breed which is at the risk of extinction. In Brazil there are less than 100 Junqueira remaining.

On May, 2005 we saw the birth of the first two *Bubalis bubalus* in the Americas, obtained from OPU/IVP vitrified embryos by Manoel Francisco de Sá Filho, one of Dr. Pietro's S. Baruselli team (USP-Pirassununga/SP). Another calf will be born in July 2005, resulting from a fresh embryo. They team consolidated new perspectives for research and commercial purposes.

Brazil has established its position in this century as the world leader in *in vitro* production of bovine embryos by ovum pick-up/i*n vitro* fertilization.

The SBTE reunite investigators from several different areas, mainly involved with bovine production. Government agencies or research institutions, as well as private companies, are coordinated by the work of our colleagues, either monitoring or executing technical and/or commercial projects (to attest the work of our community). SBTE members are active consultants in different research-supporting institutions in our country. They are responsible for important decisions that dictate the path of our research efforts. Working under the most judicious principles of honesty, they should evaluate research grant/proposals to point out the right direction the investments should go. These SBTE members will always be remembered for their impartiality and for their valuable contribution to our community.

The successful work done by the SBTE members was accomplished thanks to the high technical expertise of those involved and the investments made in our projects by the Brazilian Government or Private agencies, a similar example to what occurs in the agricultural and in the pharmaceutical industry. The development of the embryo technology wouldn't have happened without the effort, dedication, and the immeasurable courage to innovate demonstrated by the pioneers Drs. Jorge Nicolau Neto, Carlos Antonio Mondino Silva, Jorge Roberto Chebel, Walter de Pádua Becker, Teodoro Romano Vaske, Roberto Mozer de Abreu, Aurelino Menarim Júnior, Assis Roberto de Bem, Altino Pires de Almeida Filho, João Junqueira Fleury, João Roberto Basile, and their followers: Johannes Wopereis, Celso Martins de Medeiros, Múcio Teixeira Alvim, Evâneo Nogueira Coelho, José Luciano Reis Lara**,** Cyro da Porciuncula Dias da Costa, Douglas Brasil Gaeti, Carlos Fernando Marins Rodrigues, Aurenilda Silva, Carlos Alberto Zanenga, Milton Moreira, Célio Freitas, José Mendes de Oliveira, Sonia Maria Nogueira Gaspar Paggiaro, Jairo Luiz Ramos Neto, Agnaldo Barbosa Sena, José Abdo de Andrade Hellú, Takeshi Morita, Evandro Palhares Dias**,** Luis Felipe Cintra Mello, Merlison Figueiredo Pedroso, Miriam Vilela Duarte, Regivaldo Vieira de Sousa, Rubens César Pinto da Silva and Álvaro Leme, to name a few. They built the base of trust and credibility of the ET programs along with the breeders and scientific community, covering the path of both basic and applied research fields.

SBTE has reached maturity thanks to the invaluable contribution from each member, past

directors and committee members. Our mission is based on ethical work and respect for the environment, a practice held by all the colleagues since the beginning. Promoting the participation of young scientists in all events was a wise decision showing that SBTE has their eyes wide open to the future. The "student competition" session in our events, with presentations of their first research work represents " the embryo " which will result in futures researchers, the future of a scientific community.

That is why we are so confident in the future of Brazilian scientific research provided that SBTE keeps receiving the support and dedication from all professionals and graduates, from private Companies, from governmental and non-governmental institutions and from the emergent teams. Thus we can continue our progress, to keep alive a scientific and social society that promotes and maintain human relations.

Looking back at the picture of the 80's and remembering SBTE founders and those that followed them with the same passion and respect for the animals, I think we can summarize all **studies with oocyte and embryo cryopreservation, OPU/IVP in *Bos taurus taurus*, *Bos taurus indicus* and *Bubalus bubalis*, the new concepts about oocytes, embryos and superovulation in mares**, and more recently, with the non surgical collection of caprine and ovine embryos, as a result of the **"Knowledge made in Brazil",** our contribution to science. Today we can celebrate the SBTE maturity and for that reason, we can raise a toast to honor our achievements.

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* Voluntary collaborators

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	Assis Roberto de Bem	Assis Roberto de Bem
	Roberto Jorge Chebel	Roberto Jorge Chebel
	Jorge Nicolau	Jorge Nicolau
	Neuci Adalto Vinha	Neuci Adalto Vinha
	Walter Antonio de Pádua Becker	Walter Antonio de Pádua Becker
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	Claudemir de Carvalho	Claudemir de Carvalho
	Theodoro Romano Vaske	Theodoro Romano Vaske

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Buratini Jr., J., Castilho A.C.S., Teixeira A.B., Costa I.B., Pinto M.G.L., Giometti I.C., Glapinski V.F., Nogueira, M.F.G., Barros, C.M. & Price C.A. 2005. **Advances in the understanding of follicle developmental physiology.** *Acta Scientiae Veterinariae*. 33 (Suppl 1): 55-68.

ADVANCES IN THE UNDERSTANDING OF FOLLICLE DEVELOPMENTAL PHYSIOLOGY

Buratini Jr., J.[1]; Castilho, A.C.S.[1]; Teixeira, A.B.[3]; Costa, I.B.[3]; Pinto, M.G.L.[3]; Giometti, I.C.[3]; Glapinski, V.F.[2]; Nogueira, M.F.G.[3]; Barros, C.M.[2]; Price, C.A.[4]

Departments of [1]Physiology and [2]Pharmacology, IB, UNESP, Botucatu, SP, Brazil; [3]Department of Animal Reproduction and Veterinary Radiology, FMVZ, UNESP, Botucatu, SP, Brazil; [4]Centre de Recherche en Reproduction Animale, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Qc, Canada. buratini@ibb.unesp.br

Abstract

This review summarizes the main mechanisms underlying preantral and antral follicle development. Special attention is given to regulatory paracrine mechanisms in the preantral phase, and to dominant follicle selection in the antral phase. Evidence for roles of fibroblast growth factors (FGFs) in the regulation of follicle development are highlighted.

Introduction

The majority of the oocytes present in the ovary at birth are never released at ovulation (Yang et. al., 1998). Therefore, assisted reproductive technologies such as superovulation/ET and IVM/IVF have been developed and improved in order to maximize the reproductive potential of superior or endangered females. In cattle, hormone protocols that control follicle development and luteal function allow timed AI and synchronization of recipients for ET, thus enhancing reproductive efficiency (Barros & Ereno, 2004; Bó et al, 2004). However, advances in assisted reproductive technologies require a better understanding of ovarian physiology. Despite the huge amount of information produced over the last two decades, a full understanding of mechanisms underlying follicle development has yet to be achieved. The regulation of follicle development is complex, involving endocrine, paracrine and autocrine factors which are orchestrated in a stage specific manner to control a variety of processes including follicular cell proliferation and differentiation, steroidogenesis, angiogenesis/ vascularization, basal membrane and extracellular matrix remodeling and atresia/apoptosis (Webb et al., 2003; Silva & Price et al., 2000; Acosta & Miyamoto, 2004; Rodgers et al., 2003; Fortune et al., 2004). The objective of this review is to summarize the main mechanisms underlying preantral and antral follicle development, pointing out evidence of the involvement of fibroblast growth factors (FGFs). With regard to the regulation of antral follicle development, special attention is also given to the controversial role of LH receptors (LHR) in dominant follicle selection.

Preantral follicle development

The role of gonadotropins in the control of preantral follicle development is controversial. FSH receptors (FSHR) can be detected in bovine primary follicles (Wandji et al., 1992) and stimulation of preantral follicle development in vitro can be achieved by adding FSH to the culture medium. However,

FSH is considered to play a permissive rather than regulatory role at this stage of development (Gutierrez et al., 2000; McNatty et al., 1999; Webb et al, 2003). Instead, initiation and regulation of preantral follicle development are predominantly driven by locally produced factors (McNatty et al., 1999). The oocyte plays an active role in coordinating the proliferation and differentiation of surrounding granulosa cells (Gilchrist et al., 2004). Intercellular communication is provided by trans-zonal cytoplasmic processes (TZP), which are extensions of granulosa cells that penetrate through the zona pellucida and reach the oocyte membrane, where gap junctions allow a bi-directional transport of ions, metabolites, amino acids and small regulatory molecules (Albertini et al., 2001). Interestingly, this type of oocyte-somatic cell communication seems to be regulated during development as TZPs retract when the follicle reaches the antral stage, which is believed to be, at least in part, an effect of FSH action (Albertini et al., 2001).

Oocyte-somatic cell communication also occurs via paracrine signaling. Of several growth factors produced by either the oocyte or granulosa cells, stem cell factor (SCF; also known as kit ligand, KL) and members of the transforming growth factor-β (TGF-β) superfamily, particularly growth differentiation factor-9 (GDF-9) and bone morphogenectic protein-15 (BMP-15, also known as GDF-9B) have recently received most attention. In sheep, the KL receptor, c-kit, is localized to oocytes of growing follicles, and KL is produced by granulosa cells of primordial and primary follicles (McNatty et al., 1999). In mice, c-kit immunization abolished transition of primordial into primary follicles, and a mutation in the c-kit gene impaired activation of primordial follicles (Kissel et al., 2000; Yoshida et al., 1997), which highlight the absolute requirement of c-kit/KL interaction for preantral follicle development. Interestingly, KL stimulates DNA synthesis in granulosa cells, which is thought to be mediated by oocyte-derived mitogens since granulosa cells do not express the receptor c-kit (Yoshida et al., 1997; Otsuka & Shimasaki, 2002). Thus, under KL stimulation, the oocyte would trigger a very early signal to promote granulosa cell proliferation. In addition, the c-kit/KL system may also be involved in the differentiation of stroma cells into theca cells, as c-kit is expressed by theca cells and KL expression increases in granulosa cells at the time of theca layer formation (Parrot & Skinner, 2000).

In most species, ovarian GDF-9 and BMP-15 are exclusively expressed by the oocyte (reviewed by Juengel et al., 2004 and Shimasaki et al., 2003), however, both were also detected by PCR in granulosa cells of antral follicles in goats (Silva et al., 2005). GDF-9 expression is detectable from primordial (in ruminants; Bodensteiner et al., 1999) or primary (in mice; McGrath et al., 1995) stages of follicle development, and is essential for secondary follicle development as mice lacking the gene were found to be infertile with follicular development arrested at the primary stage (Dong et al., 1996). Furthermore, GDF-9 stimulated rat preantral follicle growth *in vivo*, likely through enhanced granulosa cell proliferation (Vitt et al., 2000ab). Like GDF-9, BMP-15 also stimulates granulosa cell proliferation and is crucial for preantral follicle development as ewes immunized against BMP-15 had follicles arrested at the primary stage (Otsuka et al., 2000; Juengel et al., 2002). Naturally occurring mutations in sheep have contributed to clarify the role of BMP-15. Inverdale ewes with a single inactive BMP-15 gene are fertile and show increased ovulation rate, whereas ewes homozygous for the inactivating mutation are sterile and display follicle development arrest at the primary stage (Galloway et al., 2000). It was suggested that reduced BMP-15 levels in heterozygous ewes would enhance follicle development due to an increase in sensitivity to FSH. In fact, BMP-15 was shown to suppress expression of FSHR and

FSH responsive genes including StAR (steroidogenic acute regulatory protein), P450scc (P450 side-chain clivage), 3β-HSD (3β-hydroxysteroid dehydrogenase), LHR and inhibin (Otsuka et al. 2001; Shimasaki et al., 2003).

GDF-9 and BMP-15 activate a receptor complex formed by two surface receptor subtypes (I and II). In mammals, seven type I and five type II receptors have been characterized. BMPR-II is the type II receptor activated by both GDF-9 and BMP-15, whereas BMPR-IB and TGFβR-I are type I receptors for BMP-15 and GDF-9, respectively. BMPR-II and -IB were localized to granulosa cells and oocytes of sheep preantral follicles, in agreement with the hypothesis that GDF-9 and BMP-15 regulate granulosa cell and oocyte function at early stages of follicle development (reviewed by Juengel et al., 2004). A negative feedback loop may exist between BMP-15 and KL. BPM-15 stimulates KL expression which in turn inhibits BMP-15 expression. However, the interruption of c-kit/KL signaling in a co-culture system (granulosa cells with oocytes) suppressed BMP-15-induced granulosa cell proliferation, suggesting that an interaction between BMP-15 and KL is important for follicle growth. (Otsuka & Shimasaki, 2002; Shimasaki et al., 2003). In contrast, GDF-9 inhibited KL expression in mouse granulosa cells (Joyce et al., 2000). Even though temporal secretion patterns of these growth factors are still unknown, it has been hypothesized that initial follicle development would be driven by the interaction of KL and BMP-15, whereas GDF-9 would be secreted a little later by fully grown oocytes to promote granulosa cell proliferation and modulate KL function (Shimasaki et al., 2003).

The insulin-like growth factor (IGF) system also appears to contribute to the regulation of preantral follicle growth through endocrine mechanisms. Although IGF-I and II are not expressed in preantral follicles, they do express IGF receptor (type 1) and IGF binding proteins (IGFBP-2 and 3), which are thought to regulate bioavailability of extraovarian IGFs (Webb et al., 2003). The effects of IGFs on preantral follicle development have varied with the culture system used, but stimulation was achieved when nearly physiological doses of insulin were used (Gutierrez et al., 2000; Fortune et al., 2004). Moreover, the importance of IGFs for early stages of follicle development was clearly demonstrated by gene knock-out experiments in mice which resulted in severely impaired preantral and early antral folliculogenesis (Elvin & Matzuk, 1998)

Role of FGFs

Several other growth factors are also involved in preantral paracrine/endocrine signaling including activin, follistatin, inhibin, epidermal growth factor (EGF) and FGFs (McNatty et al., 1999). FGF-2 (basic FGF) is certainly the most extensively investigated FGF with regard to follicle development. In the bovine ovary, FGF-2 was localized to oocytes of primordial and primary follicles, and granulosa and theca cells of growing preantral and antral follicles (Van Wezel et al., 1995). Binding studies revealed that receptors for FGF-2 are mainly localized to the granulosa cell layer (Wandji et al., 1992). FGF-2 was found to stimulate activation of primordial follicles in cultured fragments of rat ovary and promote granulosa cell proliferation (Nilsson et al., 2001; Gospodarowicz et al., 1989). However, FGF-2 had no effect on the spontaneous activation of primordial follicles that is commonly observed with culture of bovine ovarian explants (Derrar et al., 2000).

Another potentially interesting member of this family is FGF-8, first described as a crucial signaling

factor for embryo development and tumorigenesis (Crossley & Martin, 1995; Tanaka et al., 1992). In the mouse ovary, FGF-8 gene expression was found to be restricted to the oocyte (Valve et al., 1997) suggesting a potential role in signaling to follicular cells by the oocyte. There are five known FGF receptor (FGFR) genes (Kim et al., 2001; Sleeman et al., 2001), of which FGF-8 preferentially activates FGFR-4 and the 'c' splice form of FGFR-3 (Ornitz et al., 1996). However, mRNA encoding FGFR-4 or -3c were not consistently detected in the rodent ovary (Asakai et al., 1994; Puscheck et al., 1997). Recently, FGF-8 and FGFR-3c and -4 gene expression was detected in pools of primordial, primary and secondary preantral follicles in bovine fetal ovaries (Buratini et al., 2005a). FGF-8 and FGFR-3c expression occurred concurrently, and more frequently in secondary than in primordial follicles. The pattern of gene expression observed suggests that expression of FGF-8 and its receptors is developmentally regulated, and that FGFR-3c is the predominant receptor for FGF-8 in preantral follicles (Buratini et al., 2005a).

We have also detected mRNA encoding FGF-7 and -10, also known as keratinocyte growth factors 1 and 2 (KGF-1 and -2) respectively, in primordial, primary and secondary preantral follicles obtained from bovine fetuses, suggesting the participation of these FGFs in the mechanisms controlling preantral folliculogenesis (data not published).

Antral follicle development

Unlike the preantral stage, antral follicle development is critically dependent on gonadotropin support. In several domestic species, antral follicles are recruited and grow simultaneously in a follicle wave under the control of gonadotropins (Fortune et al., 2001; Ginther et al., 2001). However, it has also become clear that locally produced growth factors are key stimulatory/regulatory molecules in antral follicles acting via paracrine and endocrine mechanisms (Fortune et al., 2004; Ginther et al., 2001; Web et al., 2003). A rise in FSH plasma concentrations stimulates follicle recruitment and wave emergence (Adams et al., 1992; Fortune, 1994). In monovular species, one follicle is selected from the recruited pool and acquires ovulatory capacity while subordinate follicles undergo atresia. The selected follicle is known as the dominant follicle and plays an active role in suppressing growth of subordinate follicles through secretion of estradiol and inhibin (Fortune, 1994; Ginther et al., 1996). In cattle, follicles can reach approximately 8mm diameter independent of LH support, but growth beyond 9mm requires endogenous LH or exogenous FSH (Gong et al., 1996). Therefore, follicles are considered to be FSH-dependent until dominance occurs, after which they become LH-dependent (reviewed by Fortune et al., 2001, Ginther et al., 2001).

Granulosa cell LH receptors appear to be linked to follicle dominance. The comparison of gene expression patterns observed by in situ hybridization in recruited and selected bovine antral follicles indicated that selection is associated with the onset of LHR gene expression in granulosa cells (Bao & Garverick, 1998). This is supported by the increase in LHR mRNA detected by RT-PCR in granulosa cells of future-dominant follicles compared to subordinate follicles that occurs at or just prior to the time of deviation (Beg 2001). However, this is in contrast to a study reporting undetectable levels of LHR mRNA in granulosa cells during follicle selection by in situ hydridization (Evans & Fortune, 1997), and with unchanging levels of hCG binding to granulosa cells during the estrous cycle (Ireland

& Roche, 1983). There is also some controversy over when LHR appear in granulosa cells. LHR mRNA was detected by RT-PCR in granulosa cells of follicles of 7-8mm diameter (Beg et al., 2001), and in granulosa cells of follicles <5mm diameter (Robert et al., 2003), but were not detected before 9mm diameter by in situ hybridization (Xu et al., 1995).

Differences in the sensitivity of the techniques used to assess gene expression may contribute to the discrepancies described, above however another aspect to be considered is the transcriptional behavior of the LHR gene. The LHR gene is subject to alternative splicing which results in several mRNA isoforms (alternative transcripts) whose follicular expression patterns, and binding and functional properties are not yet fully elucidated. Several mRNA transcripts have been detected by PCR in sheep and cattle (Robert et al., 2003; Abdennebi et al., 2002), and four LHR alternative transcripts were recently detected by our laboratory in granulosa cells from bovine antral follicles by PCR of the region comprised within the end of exons 9 and 11, revealing alternative deletion of exon 10 and part of exon 11. Interestingly, all isoforms were detected in granulosa cells of follicles ³8mm, but only one of six follicles 7mm diameter showed LHR expression in granulosa cells (Nogueira et al., 2005a). Considering that follicles used in this experiment were predominantly obtained from Nelore females and that deviation occurs around 6mm at diameter in this breed (Sartorelli et al., 2005), we assume that LHR gene expression in granulosa cells was detected after dominant follicle selection in our studies. Moreover, LHR mRNA levels in granulosa cells were positively correlated with follicle diameter and intra-follicular progesterone concentrations (Nogueira et al., 2005a), and FSH increased expression of all four LHR transcripts in cultured granulosa cells (data not published) indicating that FSH induces sensitivity to LH. We have also demonstrated an alternative transcript with a deletion of exon 3 in the bovine LHR (Nogueira et al., 2005a) as previously reported in rats (Aatsinki et al., 1992).

What is the practical interest in LHR isoform expression? There is evidence that different isoforms exhibit different binding and functional activities. The full-length LHR protein binds to both LH and hCG molecules, whereas a truncated isoform lacking exon 10 binds exclusively to hCG (Gromoll et al., 2000) and the partial deletion of exon 11 leads to apparent receptor inactivity (Kawate et al., 2002). Based on this information, we tested the hypothesis that in superstimulated Nelore cows administration of both LH and hCG, as an attempt to stimulate different LHR isoforms in the follicles, would affect oocyte quality and/or increase ovulation rate, but no differences were observed in number of viable embryos or pregnancy rates of cows treated with LH only or LH and hCG (Nogueira et al., 2005b). Further studies are necessary to clarify the biological significance of LHR alternative splicing in cattle.

There is strong evidence that the IGF system may play a critical role in selection of the dominant follicle. IGFs synergize with FSH to promote follicle growth and estradiol production (Fortune et al., 2004). Although IGF-I and -II gene expression was localized to bovine granulosa and theca cells, respectively, it has been suggested that IGF-II is the major intraovarian IGF, whereas IGF-I would act in an endocrine manner (Armstrong et al., 2000; Yuan et al., 1998, Webb et al., 1999). IGF-I and -II activate IGF receptors type I and II, both present in granulosa and theca cells (Spicer et al., 2004). Total IGF levels were not different in the follicular fluid of bovine dominant versus subordinate follicles (de la Sota et al., 1996), but free IGF-I was higher in the follicular fluid of the largest follicle compared with the second largest of the same wave before differences in estradiol concentration or diameter were

observed (Beg et al., 2002). This observation agrees with a regulatory role for IGFBPs via modulation of IGF bioavailability. IGFBP-2, -3, -4 and -5 are present in bovine follicular fluid, and IGFBP-2 and -4 gene expression was localized to granulosa and theca cells, respectively (reviewed by Fortune, et al. 2001 and Webb et al., 1999). Reduced levels of IGFBP-4 were found in bovine dominant follicles compared with the two largest subordinate just 1.5 days after wave emergence (Mihm et al. 2000). There is strong evidence to suggest that reduced IGFBP-4 levels in the selected follicle is a consequence of increased IGFBP proteolytic degradation by pregnancy-associated plasma protein (PAPP-A), which was recently detected in the follicle (Mazerbourg et al., 2001). In fact, degradation of not only IGFBP-4 but also of IGFBP-5 was found to be higher in the largest follicle before the expected time of follicle deviation (Fortune et al., 2004). On the other hand, intra-follicular levels of IGFBP-2 seem to decrease as the dominant follicle develops, but later in relation to IGFBP-4 and 5 (Fortune et al., 2004). Unlike IGFBP-4 and -5, IGFBP-2 concentrations appear to be regulated at the transcriptional level by FSH, as IGFBP-2 mRNA was not detected in granulosa cells of large dominant follicles and FSH inhibited IGFBP-2 gene expression in cultured bovine granulosa cells (Armstrong et al., 1998; Webb et al., 2003).

Role of FGFs

Strong evidence indicates the involvement of FGFs in the paracrine mechanisms that regulate antral follicle development. As mentioned above, FGF-2 is the most extensively investigated FGF in follicle development. Gene and protein expression data revealed that the main site of FGF-2 production is the theca layer (Berisha et al., 2000). Although binding studies suggested that the main target for FGF-2 is the granulosa layer (Wandji et al., 1992), FGF-2 receptors were also expressed in theca cells (Berisha et al., 2004; Buratini et al., 2005b). In fact, both cell types seem to respond to FGF-2 as it was shown to induce proliferation and inhibit steroidogenesis in cultures of both granulosa (Lavranos et al., 1994; Vernon & Spicer, 1994) and theca cells (Nilsson et al., 2001; Spicer & Stewart, 1996). Since FGF-2 mRNA levels increase with follicle development in bovine theca cells, it was hypothesized that FGF-2 is specially involved in the control of final growth of preovulatory follicles by stimulation of angiogenesis and granulosa cell survival and proliferation (Berisha et al., 2004).

Like FGF-2, FGF-1 is also predominantly expressed by theca cells but is stable across stages of antral follicle development. In contrast to FGF-2, FGF-1 protein was mainly localized to the granulosa cell layer, where it is likely bound to its receptors, and to blood vessels. The regulatory function of FGF-1 has not been well characterized, but expression data coupled with effects previously described in other tissues suggest an anti-apoptotic/pro-survival role in blood vessels and granulosa cells (Berisha et al., 2004).

Data supporting a regulatory role for FGF-7 in antral follicle development in cattle have also been reported. Taken together, reports about FGF-7 gene and protein expression indicate that the theca layer is the main site of FGF-7 production (Parrot et al., 1994; Berisha et al., 2004). Like FGF-2, FGF-7 was shown to promote granulosa cell proliferation and to inhibit steroidogenesis in vitro (Parrot et al., 1994; Parrot & Skinner, 1998), which is in agreement with the predominant localization of its receptor (FGFR2b) mRNA to the granulosa layer (Parrot & Skinner, 1998; Berisha et al., 2004). These

paracrine actions appear to be modulated at the receptor level as FGF-7 mRNA levels did not vary during antral follicle development, whereas FGFR-2b gene expression was shown to increase with intrafollicular estradiol concentrations (Berisha et al., 2004). To gain more insight into FGFR-2b regulation, we have recently demonstrated that FSH, but not IGF-I, stimulates FGFR-2b gene expression in cultured bovine granulosa cells (Buratini et al., 2005c).

We have recently detected FGF-10 mRNA expression in oocytes and theca cells of bovine antral follicles. Interestingly, despite the similarity between FGF-7 and -10, our studies indicate high expression of FGF-10 and absence of FGF-7 mRNA in bovine oocytes (Buratini et al., 2004). Since granulosa cells express FGF-10 receptors (FGFR-2b; Berisha et al., 2004), our results suggest the involvement of FGF-10 in paracrine signaling from the oocyte and theca cells targeting granulosa cells. Moreover, FGF-10 mRNA levels in theca cells decreased with intrafollicular estradiol concentrations indicating that it is developmentally regulated (Buratini et al., 2004). This, coupled with the observation that FSH stimulates FGFR-2 expression in granulosa cells (Buratini et al., 2005c), suggests that FGF-10 of thecal origin specifically regulates mural granulosa cells of recently recruited antral follicles.

Gene expression data also suggest the participation of FGF-8 and its cognate receptors (FGFR-3c and FGFR-4) in the control of antral follicle development. In bovine antral follicles, we detected FGF-8 mRNA not only in oocytes but also in granulosa and theca cells (Buratini et al., 2005b), in contrast with previous studies in mice in which FGF-8 was considered to be oocyte-specific (Valve et al., 1997). FGFR-4 gene expression was exclusively detected in bovine theca cells, which is in contrast to data from the mouse showing expression only in granulosa cells (Buratini et al., 2005b; Puscheck et al., 1997). Thecal FGFR-4 mRNA levels decreased with increasing follicle diameter, suggesting that FGFR4 signaling may play a role in early thecal development/differentiation (Buratini et al., 2005b). FGFR-3c gene expression was observed in granulosa and in theca cells of bovine antral follicles. Whereas the expression of FGFR-3c in theca cells was relatively stable, granulosa cell expression increased during follicle development (based on follicle size and estradiol content), consistent with a role for FGFR-3c signaling during antral follicle growth. Functional studies revealed that FGFR-3c expression is specifically up-regulated by FSH in cultured bovine granulosa cells, but is not under IGF-1 control (Buratini et al., 2005b). These important observations suggest that FSH may sensitize granulosa cells to one or more FGFs during early growth of the antral follicle, and may permit continued growth of the follicle in the low-FSH environment that follows follicle deviation. Which ligand(s) might activate FGFR-3c and -4 in follicles remains to be clarified as, apart from FGF-8, FGF-1, -2, -4, -9 and -13 also efficiently activate FGFR-3c and -4 (Greene et al., 1998, Ornitz et al., 1996).

Concluding remarks

This review has addressed two areas of current research that should greatly increase our understanding of follicle development and lead to improved control of fertility. It is known that the acquisition of LH receptors on granulosa cells is essential for follicle maturation, and it is now appreciated that not all receptors are created equal: alternative splicing of the LHR gene leads to LHR isoforms that respond differently to LH and hCG. Given the widespread use of LH/hCG in reproductive management protocols, an understanding of LHR molecular biology is of potential practical use. Growth factors are

potent modifiers of follicle growth, and the IGF family is the most well known. In this review we propose that the FGF family also plays vital roles, and in the future, manipulation of these proteins may lead to improved control of follicle growth and oocyte quality.

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PERSPECTIVES ON THE USE OF BIOTECHNOLOGY IN EQUINE REPRODUCTION

Squires, E.L.

Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO 80523 USA, esquires@colostate.edu

Introduction

The use of embryo transfer and the development of embryo technologies have increased dramatically during the past decade. Today, the collection and transfer of fresh and cooled transported embryos is commonplace in most equine breeds. The United States and Brazil lead the world in the number of equine embryo transfers. Since 2002, the largest breed registries in the United States, the American Quarter Horse Association and the American Paint Horse Association, have allowed unlimited registration of foals from a mare during a given year using embryo transfer. This has more than doubled the number of embryo transfer performed each year in the United States. Multiple foal registration from a given mare has also increased dramatically the price of genetically superior mares. In addition, the breeder has demanded the development of techniques to improve reproductive performance of older mares and older stallions. This has stimulated further research on such techniques as superovulation, frozen embryos, in vitro fertilization, intracytoplasmic sperm injection, oocyte transfer, cloning and oocyte freezing. This review will give a perspective as to the status of each of these techniques and their practicality for the horse industry.

Key words: equine, embryo, technologies

Superovulation

Currently, the majority of embryos recovered are from spontaneously single-ovulating mares resulting in an embryo collection rate of 50% per attempt. One of the major costs of embryo transfer is maintenance of recipient mares when no embryo is available for transfer. One method to substantially decrease this cost is to increase the number of embryos recovered per donor through induction of multiple ovulations. Other advantages of increased follicular development would be for collection of oocytes to be used for in vitro fertilization or oocyte transfer. Most of the studies on induction of superovulation in mares have used an equine pituitary extract (EPE) high in follicle-stimulating hormone (FSH) content. The initial work on using EPE for induction of ovulation was conducted in pony mares in Wisconsin during both the anestrus and breeding seasons. Douglas [5] reported a 2.3 ovulation rate for mares given EPE once daily for seven days versus one ovulation for untreated controls. An average of 3 ovulations per mare was reported by Woods and Ginther [27] using EPE. Squires et al. [23]

reported 3.8 ovulations for EPE-treated mares and 2 embryos recovered per mare. This compared to 1.2 ovulations in untreated control mares and 0.65 embryos per cycle.

Numerous studies have been conducted at Colorado State University over the past two decades evaluating the response of mares to EPE. The ovulation rate has ranged from a low of 2.3 to a high of 3.9 ovulations per mare. Based on 170 mares treated with EPE at Colorado State University, an average of 3.2 ovulations was detected and 1.96 embryos was recovered per mare compared to 0.56 embryos recovered from untreated control mares. Mares respond better to EPE when given early in the cycle (day 5 vs day 12) [4].

Recently, a commercially purified equine pituitary extract product (eFSH) was made available from Bioniche Animal Health (Athens, GA, USA). Several studies have been conducted in our laboratory evaluating the response of mares administered eFSH. In the first preliminary trial, 12 mg of eFSH given twice daily followed by hCG once mares had obtained preovulatory follicles, resulted in 3.4 ovulations per mare and at 14 days after insemination, 1.8 pregnancies per mare versus 0.6 pregnancies for untreated controls [8]. In Brazil, 16 mares were used during 2 cycles [22]. The first cycle was considered a control cycle and in the second cycle, mares were given eFSH (12.5 mg bid). Embryo recovery was attempted 7 days after ovulation. In the control cycle, mares ovulated one follicle per mare and embryo recovery was 0.5 embryos per mare. In contrast, mares given eFSH had 3.6 ovulations and 1.9 embryos recovered per mare. Squires et al. [24] reported on the effect of delaying administration of hCG (coasting the follicles) for approximately 1.5 days after the end of eFSH treatment. They also tested giving 12.5 mg bid versus 25 mg once per day. A fourth treatment evaluated the possible beneficial effect of adding LH to the eFSH during the last part of the treatment period. The average number of days mares were treated was similar among groups (i.e. approximately 6 days). However, the number of preovulatory follicles after eFSH treatment was higher for mares allowed to coast prior to hCG than for the other groups. Ovulation rate was lower for mares administered eFSH once per day versus twice per day and there appeared to be no advantage of adding LH to the eFSH. Based on 73 cycles, approximately 85% of the mares had ≥2 ovulations. Overall, the embryo recovery rate did not differ among groups and was approximately 2 embryos per flush attempt.

Studies ongoing at Colorado State University are designed to evaluate the effect of administering progesterone and estradiol prior to initiation of eFSH. Embryo recovery was higher in mares that were not treated with progesterone and estradiol prior to eFSH versus those that were treated with progesterone and estradiol prior to eFSH (2.7 embryos per flush vs 1 embryo per flush, respectively). A second study evaluated the possible detrimental effects of treating a mare with eFSH for three consecutive cycles. These mares were compared to those that were administered eFSH on an every-other-cycle basis. There did not appear to be any adverse effect of treating mares with eFSH for three consecutive cycles.

One of the deterrents to the use of eFSH is the cost of administering the drug for 6 to 7 days. Therefore, a study was conducted to determine if a three-day treatment would provide the same response as a longer treatment. Although based on only 10 mares, the ovulation rate and embryo recovery rate

appeared similar between the two groups. Our current strategy for using eFSH is to examine the mare with ultrasonography beginning 5 to 7 days after ovulation. Once mares have acquired follicles in the range of 20 to 25 mm, then eFSH treatment will be initiated. Treatment continues on a daily basis until the majority of follicles are 32 to 33 mm. Treatment with eFSH is then stopped and hCG is given approximately 36 to 40 hr later. With this strategy, the average number of days that the mare has to be treated is only approximately four.

Another means of decreasing the amount of eFSH that is needed for superovulation is to use a lower dose than the 12 mg bid that is recommended. Based on studies conducted by Alvarenga et al. (unpublished), it would appear that a dose in the range of 6 to 9 mg, given twice a day, may be sufficient to induce superovulation in the majority of mares. The intent with this low-dose treatment is to provide two to three ovulations and one to two embryos per flush. The ideal scenario would be to improve embryo recovery without incurring tremendous cost. By using a short-term treatment of three or four days or possibly a low-dose treatment, the amount of eFSH can be minimized. Furthermore, the majority of breeders are not interested in obtaining four or five embryos from a given mare-stallion combination. Most of the breeders are desirous of obtaining one or two embryos per cycle so that they can change the stallion on the subsequent cycle and get different genetic combinations within a given breeding season.

Some of the potential problems with using eFSH are: failure of follicles to ovulate once they reach preovulatory size, overstimulation of the ovary and poor embryo recovery per ovulation. Ideally, one would like to recovery one embryo for every two ovulations (50% recovery per ovulation). However, there are some occasions were mares ovulate large numbers of follicles, but embryo recovery per ovulation is quite low. There are other occasions were mares develop numerous large follicles, but none of the follicles respond to hCG or GnRH by ovulating. Additional studies are needed to investigate the cause of anovulatory follicles in superovulated mares and the poor embryo recovery per ovulation in some cases.

Embryo Vitrification

The number of equine embryos frozen and transferred at a later date is a very small percentage of the total embryos transferred. Generally, with a low embryo recovery rate from client mares, extra embryos are not available for freezing. This is in contrast to the cattle industry, where approximately half of the bovine embryos collected are frozen for subsequent transfer. The use of eFSH for induction of ovulation provides extra embryos that could be used for freezing and subsequent transfer.

There are several advantages of freezing equine embryos. The ability to import or export equine embryos would greatly reduce the cost of introducing new genetics into a country. Another advantage includes preserving genetic material from a superior mare by embryo freezing. Freezing would also reduce the number of recipients needed in an embryo transfer program. If the number of recipients was

in short supply, then the embryo could be frozen and transferred at a later time, when recipients were available. Similarly, if recipients were out of synchrony with the donor mare, the embryo could be frozen and transferred at a later time.

Yamamoto [29] reported the birth of the first foal from a cryopreserved embryo. Takeda et al. [26] at Colorado State University obtained a 50% pregnancy rate from day-6 embryos transferred into recipients. A subsequent study in our laboratory clearly demonstrated the effect of embryo size on pregnancy rate. Seventeen embryos frozen in 10% glycerol were surgically transferred into uteri of recipient mares. Nine of seventeen resulted in viable pregnancies. However, eight of the nine recipients that became pregnant received early blastocysts and only one of seven expanded blastocysts transferred resulted in an embryonic vesicle. Since larger equine embryos failed to survive cryopreservation, many of the subsequent studies focused on protocols for freezing small embryos. Meira et al. [17] compared the efficacy of 11% glycerol and 11% 1,2 propanediol with embryos collected on days 6 or 7. Six of 15 (40%) morulae and early blastocysts cryopreserved in glycerol resulted in a pregnancy. None of the 15 morulae and early blastocysts frozen in 1,2 propanediol developed after transfer. Additionally, none of the 15 expanded blastocysts cryopreserved in glycerol resulted in a pregnancy.

Practitioners in Argentina successfully commercialized the use of embryo freezing [13]. They have reported a pregnancy rate of 56% for small equine embryos. Maclellan et al. [14] reported similar pregnancy rates between frozen-thawed and nonfrozen equine embryos that were <300 μ in size. Twelve of 17 frozen-thawed equine embryos resulted in a pregnancy at 16 days post-transfer compared to 14 of 20 nonfrozen, control embryos.

A typical protocol for slow-cool freezing of small embryos includes the addition of glycerol in two steps, 5% glycerol for 10 min and 10% glycerol for 20 min. Embryos are then placed into a 0.25-ml plastic straw and placed into a programmable cell freezer. They are cooled from room temperature to -6ºC at 4ºC/min, seeded, held at -6ºC for 10 min and then cooled at 0.3 to 0.5ºC/min to -33ºC before being plunged into liquid nitrogen. The slow-cool method for freezing equine embryos requires the use of a programmable cell freezer.

Another method of freezing that has been used successfully in other species is vitrification. This is defined as solidification of a solution brought about not by crystallization but by extreme elevations of viscosity during cooling. The vitrification procedure eliminates the need for expensive equipment and reduces the time associated with conventional slow-cooling methods of cryopreservation. Oberstein et al. [19] compared conventional slow-cooling to vitrification using embryos 150 to 300 μ in diameter. She found no difference in final embryo grade or percent of live cells among treatments. Recently, Eldridge-Panuska [6] evaluated pregnancy rate of frozen-thawed vitrified embryos. In this study, small embryos (<300 μ), morulae or early blastocysts and large (>300 μ) blastocysts were vitrified. Embryos were collected 8 days after administration of hCG for recovery of small embryos. Embryos were first placed into vitrification solution 1, which contained 1.4 M glycerol in phosphate-buffered saline. After 5 min, the embryo was removed to a second vitrification solution containing 1.4 M glycerol + 3.6 M ethylene glycol. After 5 min in that solution, the embryo was placed in a final vitrification solution

containing 3.4 M glycerol and 4.6 M ethylene glycol. The embryo remained in this final solution for <1 min before being loaded into a 0.5-ml straw. This straw was heat-sealed and placed into a cooled plastic goblet held vertically in liquid nitrogen within a styrofoam box. Liquid nitrogen surrounded the goblet, but only vapor was in contact with the straw. After 1 min, the goblet and straw were plunged into the liquid nitrogen. Embryos were thawed at room temperature for 10 sec before being placed in 22ºC water for 10 sec. No pregnancies resulted after transfer of blastocysts >300 μ. Transfer of morulae or early blastocysts vitrified in PBS resulted in 4 vesicles of 6 transferred. In a second experiment, embryos were vitrified in PBS with ethylene glycol and glycerol, according to the procedure in the first experiment. Pregnancy rates at 16 days were 26 of 48 (54%).

Recently in our laboratory, Hudson et al. [11] conducted a study to determine if cooling for 12 to 19 hr prior to vitrification would result in similar pregnancy rates to embryos vitrified immediately upon collection and to determine the viability of vitrified embryos from superovulated mares. Forty mares were superovulated using equine FSH. Embryos were recovered 6.5 days postovulation or 8 days after hCG. Forty morula and early blastocysts with a grade of 1 to 2 and <300 μ in diameter were randomly assigned to one of two treatments: Group 1 – washed 4 times in a commercial holding medium and then vitrified; Group 2 – washed 3 times and then stored in the same holding medium at 5 to 8ºC in a passive cooling device (Equitainer, Hamilton Thorne, Beverly, MA, USA) for 12 to 19 hr before being vitrified. Embryos were vitrified according to the procedure of Eldridge-Panuska et al. [6] To thaw, embryos were warmed by holding the straw in air at room temperature for 10 sec and then submerged in a water bath of 22ºC water for an additional 10 sec. The contents of the straw were transferred directly into a recipient that had ovulated 4 to 6 days previously. There was no difference in embryo diameter, grade or morphology score between treatment groups prior to vitrification. Pregnancy rates were not different between embryos vitrified immediately after collection (15 of 20, 75%) and embryos cooled for 12 to 19 hr prior to vitrification (13 of 20, 65%). Based on these results, small equine embryos can be stored at 5 to 8ºC for 12 to 19 hr prior to vitrification without a loss of viability.

Based on these two recent studies, small equine embryos can be vitrified and result in high pregnancy rates after nonsurgical transfer. The simplest scheme for obtaining small embryos that are morulae or early blastocysts is to flush the mare's uterus 8 days after hCG. It is likely that, as soon as the breed registries allow registration of foals from frozen-thawed equine embryos, then vitrification of embryos will become a common procedure given the many benefits of embryo freezing.

Oocyte Freezing

Preservation of genetic material from valuable mares that die unexpectedly or those that must be euthanized would be quite desirable. Although techniques have been published for cryopreservation of oocytes from a variety of species, pregnancy rates after thawing and insemination of these oocytes has been relatively low.

Studies on cryopreservation of equine oocytes are quite limited. Survival rates of 16% were

recorded for equine oocytes cryopreserved in ethylene glycol using a slow cooling protocol [9] and 17% using a vitrification procedure [10]. Hurtt et al. [12] compared the viability of immature and mature equine and bovine oocytes vitrified in ethylene glycol plus Ficoll and sucrose. Oocytes were packaged in open pulled straws. These oocytes were collected from slaughterhouse ovaries and either vitrified immediately or matured for 36 to 48 hr and then vitrified. After vitrification, equine oocytes had maturation rates to MII of 30, 40 and 45% for immature, mature and control oocytes, respectively. This compared with 60, 70 and 77% for bovine oocytes in the same treatments. Immature oocytes survive vitrification as well as mature oocytes. Based on live-dead determination, equine oocytes had 36, 33 and 43% live cells compared with bovine oocytes with rates of 46, 50 and 70% for the three treatment groups, respectively. Eighty-one percent of the equine immature oocytes matured to MII after vitrification. The percentage of equine oocytes that displayed both nuclear and cytoplasmic maturation for the three groups was 20, 30 and 45%. These authors concluded that both immature and mature equine oocytes survive cryopreservation using vitrification and open-pulled straws. However, the survival rate was lower for equine oocytes than for bovine oocytes.

Maclellan et al. [16], in our laboratory, evaluated the beneficial effects of adding sucrose or trehalose to the vitrification solution prior to vitrification of immature and mature equine oocytes. After thawing, oocytes were cultured for either 14 hr (immature oocytes) or 2 hr (mature oocytes) and then intracytoplasmic sperm injection (ICSI) was performed. Non-vitrified controls were cultured for 26 hr and then ICSI performed. All oocytes were cultured for 20 hr after ICSI. Fertilization rates were similar for vitrified vs non-vitrified controls and there did not appear to be a major difference between the two sugars. This same author [15] transferred vitrified oocytes into recipient mares that had been inseminated. Of 26 vitrified equine oocytes, seven were determined to be abnormal after thawing and were not transferred. Three pregnancies were obtained and two live foals were produced from these vitrified oocytes.

It is likely that, as progress is made with vitrification of oocytes from other species, these same procedures will be applied to the horse. It would be of great advantage to be able to collect oocytes from mares that have died and preserve these oocytes, as well as to collect oocytes from young mares as a means of banking genetic material from valuable mares.

Oocyte Transfer

Oocyte transfer is often used as a method to obtain embryos from problem mares. These mares have failed to provide embryos or pregnancies from embryo transfer. Mares enrolled in the oocyte transfer program may have ovulatory problems, persistent uterine infection, pyometra and/or torn or scarred cervix. Oocyte transfer involves collection of an oocyte from a donor mare and transfer of the oocyte into a recipient mare's oviduct. The oocyte is then matured in vivo. The recipient is inseminated so that fertilization and embryonic development occur within the oviduct and uterus.

The demand for oocyte transfer has increased over the past several years. Carnevale and Ginther

[1] reported high embryonic development rates (11 of 12, 92%) after oocytes were collected from young experimental donors and cultured in vitro prior to transfer into inseminated recipient oviducts. It wasn't until the 1990's that oocyte transfer was used clinically to obtain offspring from problem mares.

Carnevale et al. [2] recently conducted a retrospective analysis of the commercial oocyte transfer program at Colorado State University from 2000 through 2004. The objectives were to review the success of oocyte transfer in a clinical setting and determine potential factors that affect pregnancy rates. Oocytes were collected by ultrasound-guided follicular aspiration using a 5-mHz linear transducer. The majority of oocytes were collected between 20 and 24 hr after hCG administration to the donor and cultured in vitro for the completion of maturation before transfer. Oocyte recipients were cyclic or non-cyclic. Cyclic recipients were synchronized with the donor and received hCG on the same day as the donor. Recipients' oocytes were collected by follicular aspiration from the dominant follicle. Non-cyclic mares had minimal follicular activity and included anestrous, transitional and follicle-suppressed mares. Oocytes were transferred through a standing flank laparotomy similar to the procedures used for surgical embryo transfer. Recipients were inseminated before and/or directly after oocyte transfer with fresh, cooled or frozen semen. From 2000 through 2004, oocytes were collected from 86 light-horse mares of various breeds. Mean age of the donors was 19.2 ± 0.4 yr. Comparisons were made for mares <20 yr of age and those ≥20 yr of age. On the average, donors had 4.8 follicle aspirations per breeding season, with 3.7 oocytes recovered per donor. Oocytes were collected from 96% of the cycles and 77% of the follicles. Embryo development rate per transferred oocyte was 38% (207/548 at 16 days). In 44 mares, 2 oocytes were transferred into the same recipient. Forty-three percent of these recipients became pregnant. Twin pregnancies were detected in 14% of the recipients that received 2 oocytes and one vesicle was manually crushed. At 16 and 50 days, 40% (201/504) and 32% (159/504) of recipients with one or two transferred oocytes were diagnosed as pregnant. The overall embryonic loss rate between 16 and 50 days was 21%. The number of donors with at least one pregnant recipient at day 16 did not differ for mares <20 and ≥20 yr (39 of 45, 87% and 34 of 45, 76%, respectively). Pregnancy rates were similar for cyclic and non-cyclic recipients. Use of non-cyclic recipients allows for use of fewer mares and better synchronization of recipients and donors. This study identified two factors that had a possible negative effect on pregnancy rates: quality and number of sperm and age of donors. The results of this study and previously published studies from our laboratory confirm that pregnancies can be obtained from mares with reproductive abnormalities that are considered infertile using standard breeding procedures such as artificial insemination and embryo transfer.

In Vitro Fertilization

In vitro fertilization is used as a procedure to produced embryos in vitro in several species, as well as a treatment of subfertility. Unfortunately, little progress has been made in the development of techniques for in vitro fertilization using equine oocytes and sperm. In fact, it is difficult to find recent

reports on attempts to perform in vitro fertilization in the horse. Limited availability of oocytes, as well as the trend toward using intracytoplasmic sperm injection as a means of fertilization, has hampered the progress of in vitro fertilization.

Obviously, the two components of in vitro fertilization are oocyte maturation and sperm capacitation. For many years, it was difficult to determine if failure of in vitro production of equine embryos was due to poor oocyte maturation or lack of sperm capacitation. With the development of techniques for oocyte transfer, we can now evaluate effectively evaluate in vitro maturation systems for equine oocytes. We know that if in vivo-matured oocytes are collected from young mares and transferred into the oviducts of recipients that have been inseminated, a pregnancy rate of 70-80% is obtained. Scott et al. [2] collected oocytes from slaughterhouse ovaries and matured the oocytes in vitro for approximately 24 hr prior to transfer into the oviduct of inseminated mares. Embryonic development rates were only 10%. A subsequent study [20] compared embryonic development rates for oocytes transferred from ovaries shipped for 18 to 24 hr at two temperatures (12°C vs 22°C). In her study, oocytes were cultured in modified EMM1 medium containing 10 ng/ml of IGF, 100 ng/ml of EGF, 1 μg/ml of LH, 15 ng/ml of FSH, 1 μl/ml of E_2, 200 ng/ml progesterone and 10% fetal calf serum. After 24 hr of culture, multiple oocytes were selected from each temperature group and transferred into recipients. For each transfer, between 9 and 15 oocytes from each group were placed into the oviduct of estrous mares using standing flank laparotomy. Uteri of recipients were examined to determine the number of developing embryos. The percentage of oocytes that developed into embryonic vesicles on day 16 was not different between transport groups (22°C = 13 of 73, 18% vs 12°C = 11 of 73, 15%).

These studies would indicate that the procedures for in vitro maturation of equine oocytes have not been optimized. Studies are needed in which in vivo-matured oocytes are fertilized in vitro using proven techniques for sperm capacitation. The use of in vivo-collected oocytes would ensure that the oocyte is capable of being fertilized. Development of techniques to ensure that sperm are properly capacitated in vitro are desperately needed.

Intracytoplasmic Sperm Injection (ICSI)

Although standard in vitro fertilization is not an efficient process in the horse, fertilization of horse oocytes in vitro using intracytoplasmic sperm injection (ICSI) is becoming more commonplace. ICSI is being used to evaluate the ability of in vitro-matured oocytes to fertilize as well as to produce embryos for evaluation of equine embryo culture systems. Clinically, ICSI is being used to produce foals from stallions that have extremely low sperm numbers. Intracytoplasmic sperm injection requires a mature oocyte. These can be obtained by aspirating a preovulatory follicle or may be obtained by in vitro maturation of oocytes collected from small, immature follicles of slaughterhouse ovaries or aspiration of small, immature follicles using a transvaginal ultrasound probe.

The tremendous advantage of ICSI is that only small numbers of sperm are required and sperm can be either fresh, cooled or frozen. During ICSI, one sperm is picked up with a micropipette and the

sperm membrane broken to assure that it is immotile and that sperm cytosolic factors important for egg activation are released. The sperm is then injected into the cytoplasm of the mature oocyte. Squires et al. [25] reported the first foal born from ICSI in the horse. Four in vitro-matured oocytes were injected with sperm and transferred into the oviduct of recipient mares. One pregnancy was obtained which resulted in a live, healthy foal.

The success of ICSI has improved dramatically over the last several years. Choi et al. [3], using a Piezo injection system to perform ICSI, reported an 80% cleavage rate for injection of in vitro-matured oocytes. In our laboratory, ICSI is used to obtain pregnancies from stallions with poor quality semen or limited semen. Recently, in vivo-matured oocyte were obtained from experimental mares and injected with frozen-thawed sperm from a stallion that had previously died. Out of four presumptive embryos placed in the oviduct of a recipient, three developed into embryonic vesicles.

ICSI has routinely been used in our laboratory to produce pregnancies from subfertile stallions. Most of our work has been done with frozen semen, some of which is from stallions that have previously died. On other occasions, the stallion is still alive but the amount of frozen semen is quite limited. Generally, 30 to 40% of the injected oocytes that are transferred into the oviduct result in viable pregnancies that go on to produce normal, healthy foals. Ideally, one would like to culture the injected oocytes in vitro up to the blastocyst stage and then transfer the blastocyst into the recipient by nonsurgical transfer. Workers at Texas A&M University [8] found that culture of ICSI-produced embryos in DMEM/F12 in a mixed gas environment can support >35% blastocyst development. Blastocyst development rates of ICSI oocytes ranged from 23 to 43% using this DMEM/F12 system. Unfortunately, although they obtained approximately 50% pregnancy rate after transfer of in vitro-produced embryos, nearly half of these embryos are lost in early gestation. Further studies are needed to optimize the embryo culture systems needed for in vitro-produced equine embryos.

Cloning

Nuclear transfer (cloning) allows production of a foal having the same genetics as the donor. Thus, this is a method to preserve genetics of an extremely valuable mare. The demand for cloning appears to be on the increase for those clients that have valuable mares and/or stallions. Another use for cloning is to provide a genetic copy of a gelding. This clone could be left intact as a stallion and, thus, could produce sperm that passes along the traits of the original gelding.

The techniques for cloning are quite similar to those used for intracytoplasmic sperm injection. Oocytes can either be collected from animals that have died or live animals using transvaginal, ultrasound-guided follicular aspiration. Collection of oocytes from live animals can be either from small follicles (immature oocytes) or preovulatory follicles (mature oocytes). Using the same micromanipulation set-up, the oocyte is enucleated and somatic cells from the genetic donor are injected into the enucleated oocyte. These cells can either be skin cells, fetal cells or cumulus cells. A somatic cell from the donor

is selected and combined with the enucleated oocyte either by fusing the two cells using electrical pulse or by breaking the donor cell membrane and injecting the cell directly into the cytoplasm of the oocyte. The recombined oocyte containing the nucleus of the genetic donor is then artificially activated. The transferred nucleus decondenses and the oocyte begins embryonic development.

In 2003, three cloned mule foals were produced [28] and one cloned horse foal was born [7]. Three more cloned foals were born in 2005 in Italy and two at Texas A&M University [28]. Unlike cattle, it appears that foals can be born without any assistance and essentially all of the foals born to date have been normal. Unfortunately, many of the reconstructed embryos that are transferred into recipients result in embryonic loss. Woods et al. [28] reported the establishment of 7 pregnancies from the transfer of 62 oocytes, but all pregnancies were lost before day 80. This group used in vitro-matured oocytes and cells from a mule fetus, rather than an adult mule. The cloned horse produced in Italy in 2003 [7] was produced using adult fibroblast, in vitro-matured oocytes and embryo culture to the blastocyst stage before transcervical transfer to the uterus of recipient mares. In that report, 841 recombined oocytes were cultured, 22 blastocysts developed (3%). Seventeen blastocysts were transferred and 4 pregnancies resulted (24% pregnancy rate after transfer). Of the four pregnancies, two were lost around day 30, one was lost at 6 months of gestation and one was carried to term for a normal birth. For the foal born in Italy in 2005, 200 recombined oocytes were cultured, resulting in 34 embryos (17%) and 3 pregnancies (9% pregnancy rate), of which one produced a viable foal. At Texas A&M University in 2005 [8], 423 recombined oocytes resulted in 6 blastocysts (1.4%) and one pregnancy (17% pregnancy rate), which produced a viable foal. In a second study by this same group, 144 recombined oocytes resulted in 8 blastocysts (6%). Five were transferred for 3 pregnancies (60%), of which one produced a viable foal.

It is obvious that the in vitro blastocyst development rate for cloned embryos is much lower than that for ICSI embryos (1 to 17% vs 25 to 30%). The cloned equid foals have all been reported healthy at birth and have developed normally after birth. There is at least one commercial company that is attempting equine cloning for clients. As the efficiency of equine cloning becomes better, it is likely that there will be a greater demand for this service. It will be interesting to see whether the breed associations will allow registrations of animals that have been cloned. Currently, the American Quarter Horse Association, which is our largest breed in the USA, has outlawed the registration of foals born by cloning. Our advice is for clients to obtain cells from valuable animals and bank these cells for subsequent use. Cell banking allows a client to make a decision in the future as to whether to use these cells to produce a cloned foal.

It remains to be seen as to how exact a cloned foal is to the original horse. Although the reconstructed embryo has the nuclear DNA of the genetic donor, the mitochondrial DNA of the recipient oocyte may have some effect on the traits of the foal. Furthermore, the phenotype of the new foal will be affected by the environment, both within the uterus and postnatally. Lastly, the development of the genetic foal will be influenced by gene expression. The client should be made aware of these possibilities prior to deciding whether or not to invest the tremendous funds needed for cloning of their horse.

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EVOLUTION OF TRANSGENIC FARM ANIMALS

Niemann, H.; Kues, W.; Carnwath, J.W.

Department of Biotechnology, Institute for Animal Breeding (FAL), Mariensee, 31535 Neustadt, Germany, niemann@tzv.fal.de

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Summary

Pronuclear microinjection of deoxyribonucleic acid (DNA) has been the standard method for producing transgenic animals. This technique is now being replaced by more efficient protocols based on somatic nuclear transfer that also permit targeted genetic modifications. Lentiviral vectors and small interfering ribonucleic acid technology (siRNA) are also becoming important tools for transgenesis. Transgenic farm animals are important in human medicine as sources of biologically active proteins, as donors in xenotransplantation, and for research in cell and gene therapy. Typical agricultural applications include improved carcass composition, lactational performance and wool production, as well as enhanced disease resistance and reduced environmental impact. Product safety can be ensured by standardisation of procedures and monitored by polymerase chain reaction and array technology. As sequence information and genomic maps of farm animals are refined, it becomes increasingly practical to remove or modify individual genes. This approach to animal breeding will be instrumental in meeting global challenges in agricultural production in the future.

Keywords

Agricultural application – Environmental benefit – Farm animal – Gene pharming – Lentiviral vector – Microinjection – Nuclear transfer – Small interfering ribonucleic acid – Transgenic – Xenotransplantation.

Introduction: evolution of transgenic technologies

The first transgenic livestock were produced almost 20 years ago by microinjection of foreign deoxyribonucleic acid (DNA) into the pronuclei of zygotes [33]. However, as microinjection has several significant shortcomings – including low efficiency, random integration and variable expression patterns related to the site of integration – research has focused on alternative methodologies for improving efficiency of generating transgenic livestock (Table 1). These methodologies include:

- sperm-mediated DNA transfer [12, 54, 55]
- intracytoplasmic injection of sperm heads carrying foreign DNA [71, 72]
- injection or infection of oocytes and/or embryos by different types of viral vectors [11, 35, 37]
- ribonucleic acid (RNA) interference technology (small interfering RNA: siRNA) [14]
- nuclear transfer [5, 13, 21, 53, 86].

Table 1. Evaluation of gene transfer methodology

	Microinjection	Somatic NT	TG spermatogonia	Lentivirus	SMGT
Animal Usage	high	low	low	low	low
Site of Integration	Random	Prescreened	Random	Random	Random
Integration Prescreening	No	Yes	Yes	No	No
Homologous Recombination	No	Possible	Not yet	No	No
Multiple Transgenes	Inefficient	Yes	Not yet	Yes	Inefficient
Max. Construct Size Limitation	artificial chromosomes	transfection limited/artif.chrom	transfection limited	~ 5 kb	?
Founder Mosaicism	Yes	No	No	Yes	No/?
Technical Difficulty	😐	☹	☹	☺	☺

TG: transgenic; all grading is based on the fully matured technology.

To date, somatic nuclear transfer, which has been successful in ten species – albeit at low efficiency [47, 103] – holds the greatest promise for significant improvements in the generation of transgenic livestock (Table 1). Further qualitative improvements may be derived from technologies that allow precise modifications of the genome, including targeted chromosomal integration by site-specific DNA recombinases, such as Cre or flippase (FLP), or methods that allow temporally and/or spatially controlled transgene expression [9, 45]. The first genomes of farm animals (cattle, chicken) were sequenced and annotated in 2004 [2, 3]. Thus, after approximately 7,000 years of domestic animal selection [16] based on the random mutations caused by radiation and oxidative injury to the genome, technology is now available to introduce or remove known genes with known functions.

Despite the inherent inefficiency of microinjection technology, a broad spectrum of genetically modified large animals has been generated for applications in agriculture and biomedicine (Table 2). The use of transgenic livestock for 'gene pharming' has now reached the level of commercial exploitation [47]. This paper provides a brief summary of the current status of transgenic animal production and look at future implications. The authors focus on large domesticated species and do not cover the recent developments in poultry breeding or aquaculture.

Biomedical applications of transgenic domestic animals

Pharmaceutical production in the mammary gland of transgenic animals

Gene 'pharming' entails the production of recombinant biologically active human proteins in the mammary glands of transgenic animals. This technology overcomes the limitations of conventional and recombinant production systems for pharmaceutical proteins [63, 82] and has advanced to the stage of commercial application [27, 107]. The mammary gland is the preferred production site, mainly because of the quantities of protein that can be produced in this organ using mammary gland-specific promoter

elements and established methods for extraction and purification of that protein [63, 82].

Table 2. Current status of transgenesis in livestock

	Early exp. phase	Experimental stage	Advanced exp. stage	Transition expt./pract. use	Practical use
Biomedicine					
Gene Pharming	■	■	■	■	■
Antibody Production	■	■	■		
Xenotransplantation					
- solid organs	■	■	■		
- cells/tissues	■	■	■	■	■
Blood replacement	■				
Disease model	■	■			
Agriculture					
Carcass composition					
- leaner meat	■	■	■		
- healthful lipids	■	■			
Lactational performance	■	■	■		
Environmental impact	■	■	■	■	
Wool production	■	■			
Disease resistance	■	■			
Fertility	■				

Guidelines developed by the Food and Drug Administration (FDA) in the United States of America (USA) require monitoring of the animals' health, sequence validation of the gene construct, characterisation of the isolated recombinant protein, and monitoring of the genetic stability of the transgenic animals over several generations. This has necessitated, for example, the use of animals from scrapie-free countries (New Zealand) and the maintenance of production animals under strict hygienic conditions.

Products derived from the mammary gland of transgenic goats and sheep, such as antithrombin III (ATIII), a-antitrypsin or tissue plasminogen activator (tPA), have progressed to advanced clinical trials [47]. Phase III trials for a recombinant human ATIII product have been completed and an application

has been filed for European Market Authorisation. The protein is expected to be registered and on the market by the end of 2005. The product is employed for the treatment of heparin-resistant patients undergoing cardiopulmonary bypass procedures. At the same company that manufactures this product, 11 transgenic proteins have been expressed in the mammary gland of transgenic goats at more than one gram per litre. The enzyme a-glucosidase from the milk of transgenic rabbits has orphan drug status and has been successfully used for the treatment of Pompe's disease [94] (in the USA the term 'orphan drug' refers to a product that treats a rare disease affecting fewer than 200,000 Americans). Similarly, recombinant C1 inhibitor produced in the milk of transgenic rabbits has completed phase III trials and is expected to receive registration within the next 24 months. A topical antibiotic against *Streptococcus mutans*, for prevention and treatment of dental caries, has completed phase III trials and should be launched shortly. The global market for recombinant proteins from domestic animals is expected to exceed US$1 billion in 2008 and to reach US$18.6 billion in 2013.

Despite these remarkable achievements, some gene constructs have failed to produce economically significant amounts in the milk of transgenic animals, indicating that the technology needs further refinements to achieve high-level expression. This is particularly true for genes that have complex regulation, such as those coding for erythropoietin or human clotting factor VIII [36, 41, 62, 67]. With the advent of transgenic crops that produce pharmacologically active proteins, there is now an array of recombinant technologies that will allow selection of the most appropriate production system for each required protein [58]. The production of edible vaccines in transgenic crop plants against, for example, foot and mouth disease, might become an important application for animal health [102].

Antibody production in transgenic animals

Numerous monoclonal antibodies are being produced in the mammary gland of transgenic goats [63]. Cloned transgenic cattle produce a recombinant bispecific antibody in their blood [31]. Purified from serum, the antibody is stable and mediates target cell-restricted T cell stimulation and tumour cell killing. An interesting new development is the generation of trans-chromosomal animals. A human artificial chromosome containing the complete sequences of the human immunoglobulin heavy and light chain loci was introduced into bovine fibroblasts, which were then used in nuclear transfer. Trans-chromosomal bovine offspring were obtained that expressed human immunoglobulin in their blood. This system could be a significant step forward in the production of human therapeutic polyclonal antibodies [51]. Further studies will show whether the additional chromosome will be maintained over future generations and how stable expression will be.

Blood replacement

Functional human haemoglobin has been produced in transgenic swine. The transgenic protein could be purified from the porcine blood and showed oxygen-binding characteristics similar to natural human haemoglobin. The main obstacle was that only a small proportion of porcine red blood cells contained the human form of haemoglobin [90]. Alternative approaches to produce human blood

substitutes have focused on the chemical cross-linking of haemoglobin to the superoxide-dismutase system [20].

Xenotransplantation of porcine organs to human patients

Today more than 250,000 people are alive only because of the successful transplantation of an appropriate human organ (allotransplantation). However, progress in organ transplantation technology has led to an acute shortage of appropriate organs, and cadaveric or live organ donation does not meet the demand in Western societies. To close the growing gap between demand and availability of appropriate human organs, porcine xenografts from domesticated pigs are considered to be the best alternative [47, 77]. Essential prerequisites for successful xenotransplantation are:

– overcoming the immunological hurdles
– preventing the transmission of pathogens from the donor animal to the human recipient
– ensuring the compatibility of the donor organs with human anatomy and physiology.

The major immunological obstacles in porcine-to-human xenotransplantation are:

– hyperacute rejection (HAR)
– acute vascular rejection (AVR)
– cellular rejection, and eventually
– chronic rejection [101].

Hyperacute rejection occurs within seconds or minutes, when, in the case of a discordant organ (e.g. in transplanting from pig to human), pre-existing antibodies react with antigenic structures on the surface of the porcine cells and activate the complement cascade; in other words, the antigen–antibody complex triggers formation of the membrane attack complex. Induced xenoreactive antibodies are thought to be responsible for AVR, which occurs within days after transplantation of a xenograft; disseminated intravascular coagulation (DIC) is a predominant feature of AVR [17, 52]. Human thrombomodulin and heme-oxygenase 1 are crucially involved in the etiology of DIC and are targets for future transgenic modifications to improve the long-term survival of porcine xenografts by creating multi-transgenic pigs.

When using a discordant donor species such as the pig, overcoming HAR and AVR are the pre-eminent goals. Two main strategies have been successfully explored for long-term suppression of HAR: synthesis of human regulators of complement activity (RCAs) in transgenic pigs [18, 77] and the knockout of α-gal epitopes, the antigenic structures on the surface of the porcine cells that cause HAR [52, 74, 105]. Survival rates, after the transplantation of porcine hearts or kidneys expressing transgenic RCA proteins to immunosuppressed non-human primates, reached 23 to 135 days, indicating that HAR can be overcome in a clinically acceptable manner [4]. The successful xenotransplantation of porcine organs with a knockout of the 1,3-α-galactosyltransferase gene, eliminating the 1,3-α-gal-epitopes produced by the 1,3-α-galactosyltransferase enzyme, has recently been demonstrated. Upon transplantation of porcine hearts or kidneys from these α-gal-knockout pigs to baboons, survival rates reached two to six months [52, 105]. A particularly promising strategy to enhance long-term graft tolerance is the induction of permanent chimerism via intraportal injection of embryonic stem (ES) cells [28] or the co-

transplantation of vascularised thymic tissue [105].

Recent findings have revealed that the risk of porcine endogenous retrovirus transmission is negligible and show that this critical danger could be eliminated, paving the way for preclinical testing of xenografts [47]. Despite further challenges, appropriate lines of transgenic pigs are likely to be available as organ donors within the next five to ten years. Guidelines for the clinical application of porcine xenotransplants are already available in the USA and are currently being developed in other countries. The general consensus of a worldwide debate is that the technology is ethically acceptable provided that the individual's well-being does not compromise public health. Economically, xenotransplantation will be viable, as the enormous costs of maintaining patients suffering from severe kidney disease using dialysis or supporting those suffering from chronic heart disease could be reduced by a functional kidney or heart xenograft.

Farm animals as models for human diseases

Mouse physiology, anatomy and life span differ significantly from those of humans, making the rodent model inappropriate for many human diseases. Farm animals, such as pigs, sheep or even cattle, may be more appropriate models in which to study potential therapies for human diseases that require longer observation periods than those possible in mice, e.g. artherosclerosis, non-insulin-dependent diabetes, cystic fibrosis, cancer and neuro-degenerative disorders [34, 56, 70, 92]. Cardiovascular disease is an increasing health problem in aging Western societies, where coronary artery diseases account for the majority of deaths. Because genetically modified mice do not manifest myocardial infarction or stroke as a result of atherosclerosis, new animal models, such as swine that exhibit these pathologies, are needed to develop effective therapeutic strategies [32, 80]. An important porcine model has been developed for the rare human eye disease retinitis pigmentosa (PR) [73]. Patients with PR suffer from night blindness early in life, a condition attributed to a loss of photoreceptors. Transgenic pigs that express a mutated rhodopsin gene show great similarity to the human phenotype and effective treatments are being developed [61].

The pig could be a useful model for studying defects of growth-hormone releasing hormone (GHRH), which are implicated in a variety of conditions such as Turner syndrome, hypochrondroplasia, Crohn's disease, intrauterine growth retardation or renal insufficiency. Application of recombinant GHRH and its myogenic expression has been shown to alleviate these problems in a porcine model [26]. Development of further ovine and porcine models of human diseases is underway [29].

An important aspect of nuclear-transfer-derived large animal models for human diseases and the development of regenerative therapies is that somatic cloning per se does not result in shortening of the telomeres and thus does not necessarily lead to premature ageing [85]. Telomeres are highly repetitive DNA sequences at the ends of the chromosomes that are crucial for their structural integrity and function and are thought to be related to lifespan. Telomere shortening is usually correlated with severe limitation of the regenerative capacity of cells, the onset of cancer, ageing and chronic disease with significant impacts on human lifespans [85]. Expression of the enzyme telomerase, which is primarily responsible for the formation and rebuilding of telomeres, is suppressed in most somatic tissues post-natally. Recent

studies have revealed that telomere length is established early in pre-implantation development by a specific genetic programme and correlates with telomerase activity [84].

Transgenic animals in agriculture

Carcass composition

Transgenic pigs bearing a human metallothionein promoter/porcine growth-hormone gene construct showed significant improvements in economically important traits such as growth rate, feed conversion and body fat/muscle ratio without the pathological phenotype known from previous growth hormone constructs [69, 78]. Similarly, pigs transgenic for the human insulin-like growth factor-I had ~30% larger loin mass, ~10% more carcass lean tissue and ~20% less total carcass fat [79]. The commercialisation of these pigs has been postponed due to the current lack of public acceptance of genetically modified foods.

Recently, an important step towards the production of more healthful pork has been made by the creation of the first pigs transgenic for a spinach desaturase gene that produces increased amounts of non-saturated fatty acids. These pigs have a higher ratio of unsaturated to saturated fatty acids in striated muscle, which means more healthful meat since a diet rich in non-saturated fatty acids is known to be correlated with a reduced risk of stroke and coronary diseases [66, 83].

Lactation

The physicochemical properties of milk are mainly due to the ratio of casein variants, making these a prime target for the improvement of milk composition. Transgenic mice have been developed with various modifications demonstrating the feasibility of obtaining significant alterations in milk composition in larger animals, but at the same time, showing that unwanted side effects can occur [50, 89].

Dairy production is an attractive field for targeted genetic modification [44, 106]. It may be possible to produce milk with a modified lipid composition by modulating the enzymes involved in lipid metabolism, or to increase curd and cheese production by enhancing expression of the casein gene family in the mammary gland. The bovine casein ratio has been altered by over-expression of beta- and kappa-casein, clearly underpinning the potential for improvements in the functional properties of bovine milk [8]. Furthermore, it may be possible to create ‘hypoallergenic’ milk by knockout or knockdown of the (-lactoglobulin gene; to generate lactose-free milk by a knockout or knockdown of the a-lactalbumin locus, which is the key molecule in milk sugar synthesis; to produce ‘infant milk’ in which human lactoferrin is abundantly available; or to produce milk with a highly improved hygienic standard by increasing the amount of lysozyme. Lactose-reduced or lactose-free milk would render dairy products suitable for consumption by the large numbers of adult humans who do not possess an active intestinal lactase enzyme system. Although lactose is the main osmotically active substance in milk and a lack thereof could interfere with milk secretion, a lactase construct has been tested in the mammary gland of transgenic mice. In hemizygous mice, this reduced lactose contents by 50% to 85% without altering

milk secretion [43]. Unfortunately, mice with a homozygous knockout for a-lactalbumin could not nurse their offspring because of the high viscosity of their milk [89]. These findings demonstrate the feasibility of obtaining significant alterations in milk composition by applying the appropriate strategy. In the pig, transgenic expression of a bovine lactalbumin construct in sow milk has been shown to result in higher lactose contents and greater milk yields, which correlated with improved survival and development of piglets [100]. Any increased survival of piglets at weaning would provide significant commercial advantages to the producer.

Wool production

Transgenic sheep carrying a keratin-IGF-I construct showed that expression in the skin and the amount of clear fleece was about 6.2% greater in transgenic than in non-transgenic animals. No adverse effects on health or reproduction were observed [22, 23]. Approaches designed to alter wool production by transgenic modification of the cystein pathway have met with only limited success, although cystein is known to be the rate-limiting biochemical factor for wool growth [96].

Environmentally friendly farm animals

Phytase transgenic pigs have been developed to address the problem of manure-related environmental pollution. These pigs carry a bacterial phytase gene under the transcriptional control of a salivary-gland-specific promoter, which allows the pigs to digest plant phytate. Without the bacterial enzyme, phytate phosphorus passes undigested into manure and pollutes the environment. With the bacterial enzyme, fecal phosphorus output was reduced by up to 75% [30]. Developers expect these environmentally friendly pigs to enter commercial production in Canada within the next few years.

Transgenic animals and disease resistance

Transgenic strategies to increase disease resistance

In most cases, susceptibility to pathogens originates from the interplay of numerous genes; in other words, susceptibility to pathogens is polygenic in nature. Only a few loci are known that confer resistance against a specific disease. Transgenic strategies to enhance disease resistance include the transfer of major histocompatibility-complex genes, T-cell-receptor genes, immunoglobulin genes, genes that affect lymphokines or specific disease-resistance genes [64]. A prominent example for a specific disease resistance gene is the murine Mx-gene. Production of the Mx1-protein is induced by interferon and was discovered in inbred mouse strains that are resistant to infection with influenza viruses [88]. Microinjection of an interferon- and virus-inducible Mx-construct into porcine zygotes resulted in two transgenic pig lines that expressed the Mx-messenger RNA (mRNA); unfortunately, no Mx protein could be detected [65]. Recently the bovine MxI gene was identified and shown to confer antiviral activity as a transgenic construct in Vero cells [6].

Transgenic constructs bearing the immunoglobulin-A (IgA) gene have been successfully introduced

into pigs, sheep and mice in an attempt to increase resistance against infections [57]. The murine IgA gene was expressed in two transgenic pig lines, but only the light chains were detected and the IgA-molecules showed only marginal binding to phosphorylcholine [57]. High levels of monoclonal murine antibodies with a high binding affinity for their specific antigen have been produced in transgenic pigs [97].

Attempts to increase ovine resistance to Visna virus infection via transgenic production of Visna envelope protein have been reported [15]. The transgenic sheep developed normally and expressed the viral gene without pathological side effects. However, the transgene was not expressed in monocytes, the target cells of the viral infection. Antibodies were detected after an artificial infection of the transgenic animals [15].

It has also proved possible to induce passive immunity against transmissible gastroenteritis (TGEV), an economically important porcine disease in a transgenic mouse model [10]. The transgenic mice secreted a recombinant antibody in milk that neutralised the corona virus responsible TGEV and conferred resistance against TGEV. Strong mammary-gland-specific expression was achieved for the entire duration of lactation. Verification of this work in transgenic pigs is anticipated in the near future.

Knockout of the prion protein is the only secure way to prevent infection and transmission of spongiform encephalopathies like scrapie or bovine spongiform encephalopathy [98]. The first successful targeting of the ovine prion locus has been reported; however, the cloned lambs carrying the knockout locus died shortly after birth [24]. Cloned cattle with a knockout for the prion locus have also been generated [19]. Transgenic animals with modified prion genes will be an appropriate model for studying the epidemiology of spongiform encephalopathies in humans and are crucial for developing strategies to eliminate prion carriers from farm animal populations.

Transgenic approaches to increase disease resistance of the mammary gland

The levels of the anti-microbial peptides lysozyme and lactoferrin in human milk is many times higher than in bovine milk. Transgenic expression of the human lysozyme gene in mice was associated with a significant reduction of bacteria and reduced the frequency of mammary gland infections [59, 60]. Lactoferrin has bactericidal and bacteriostatic effects, in addition to being the main iron source in milk. These properties make an increase in lactoferrin levels in bovine transgenics a practical way to improve milk quality. Human lactoferrin has been expressed in the milk of transgenic mice and cattle at high levels [46, 76] and is associated with an increased resistance against mammary gland diseases [93]. Lycostaphin has been shown to confer specific resistance against mastitis caused by *Staphylococcus aureus*. A recent report indicates that transgenic technology has been used to produce cows that express a lycostaphin gene construct in the mammary gland, thus making them mastitis-resistant [95].

Emerging transgenic technologies

Lentiviral transfection of oocytes and zygotes

Recent research has shown that lentiviruses can overcome previous limitations of viral-mediated

gene transfer, which included the silencing of the transgenic locus and low expression levels [104]. Injection of lentiviruses into the perivitelline space of porcine zygotes resulted in a very high proportion of piglets that carried and expressed the transgene. Stable transgenic lines have been established by this method [36]. The generation of transgenic cattle by lentiviruses requires microinjection into the perivitelline space of oocytes and has a lower efficiency than that obtained in pigs [37]. Lentiviral gene transfer in livestock promises unprecedented efficiency of transgenic animal production. Whether the multiple integration of lentiviruses into the genome is associated with unwanted side-effects like oncogene activation or insertional mutagenesis remains to be investigated.

Chimera generation via injection of pluripotent cells into blastocysts

Embryonic stem cells with pluripotent characteristics have the ability to participate in organ and germ cell development after injection into blastocysts or by aggregation with morulae [81]. True ES cells (that is, those able to contribute to the germ line) are currently only available from inbred mouse strains [48]. In mouse genetics, ES cells have become an important tool for generating gene knockouts, gene knockins and large chromosomal rearrangements [25]. Embryonic stem-like cells and primordial germ cell cultures have been reported for several farm animal species, and chimeric animals without germ line contribution have been reported in swine [1, 87, 99] and cattle [13]. Recent data indicate that somatic stem cells may have a much greater potency than previously assumed [42, 49]. Whether these cells will improve the efficiency of chimera generation or somatic nuclear transfer in farm animals has yet to be shown [48].

Culture of spermatogonia and transplantation into recipient males

Transplantation of genetically altered primordial germ cells into the testes of host male animals is an alternative approach to generating transgenic animals. Initial experiments in mice showed that the depletion of endogenous spermatogenesis by treatment with busulfan prior to transplantation is effective and compatible with re-colonisation by donor cells. Recently, researchers succeeded in transmitting the donor haplotype to the next generation after germ-cell transplantation [38]. The major obstacles to this strategy are the lack of efficient *in vitro* culture methods for primordial germ/prospermatogonial cells, and the limitations this imposes on gene transfer techniques for these cells.

Ribonucleic acid interference (siRNA)

Ribonucleic acid interference is a conserved post-transcriptional gene regulatory process in most biological systems, including fungi, plants and animals. Common mechanistic elements include siRNAs with 21 to 23 nucleotides, which specifically bind complementary sequences on their target mRNAs and shut down expression. The target mRNAs are degraded by exonucleases and no protein is translated [75]. The RNA interference seems to be involved in gene regulation by controlling/suppressing the translation of mRNAs from endogenous viral elements.

The relative simplicity of active siRNAs has facilitated the adoption of this mechanism to generate transient or permanent knockdowns for specific genes. For transient gene knockdown, synthetic siRNAs can be transfected into cells or embryonic stages. For stable gene repression, the siRNA sequences must be incorporated into a gene construct [14]. The conjunction of siRNA and lentiviral vector technology may soon provide a method with high gene transfer efficiency and highly specific gene knockdown for livestock.

Safety aspects and outlook

Biological products from any animal source are unique, and must be handled in a different manner from chemically synthesised drugs to assure their safety, purity and potency. Proteins derived from living systems are heat labile, subject to microbial contamination, can be damaged by shear forces and have the potential to be immunogenic and allergenic. In the USA, the FDA has developed guidelines to assure the safe commercial exploitation of recombinant biological products. A crucial consideration with animal-derived products is the prevention of transmission of pathogens from animals to humans [47]. This requires sensitive and reliable diagnostic and screening methods for the various types of pathogenic organisms. Furthermore, it should be kept in mind that all transgenic applications of farm animals will require strict standards of 'genetic security' and reliable, sensitive methods for molecular characterisation of the products. A major contribution towards the goal of well-defined products will come from matrix-assisted laser desorption/ionisation time-of-flight spectometry (MALDI-TOF) [40, 91]. Meanwhile improvements in RNA isolation and unbiased global amplification of picogram amounts of mRNA enable researchers to analyse RNA even from single embryos [7]. This technology can offer insights into the entire transcriptome of a transgenic organism and thereby ensure the absence of unwanted side-effects [40, 91]. Rigorous control is also required to maintain the highest possible levels of animal welfare in cases of genetic modification.

Conclusion

Throughout history, animal husbandry has made significant contributions to human health and well-being. The convergence of recent advances in reproductive technologies (*in vitro* production of embryos, sperm sexing, somatic nuclear transfer) with the tools of molecular biology (gene targeting and array analysis of gene expression) adds a new dimension to animal breeding [68]. Major prerequisites for success and safety will be the continuous refinement of reproductive biotechnologies and a rapid completion and refinement of genomic sequencing projects in livestock. The authors anticipate that within the next five to seven years genetically modified animals will play a significant role in the biomedical arena, in particular via the production of valuable pharmaceutical proteins and the supply of xenografts (Table 2). Agricultural applications are already being prepared [39], but general public acceptance may take as long as ten years to achieve. As the complete genomic sequences of all farm animals become available, it will be possible to refine targeted genetic modification in animal breeding and to develop strategies to cope with future challenges in global agricultural production.

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TECHNICAL AND BIOLOGICAL ASPECTS OF ULTRASOUND-GUIDED TRANSVAGINAL OOCYTE RETRIEVAL IN THE COW: AN OVERVIEW

Bols, P.E.J.[1]; Leroy, J.L.M.R.[2]; Viana, J.H.M.[3]

[1]Laboratory for Veterinary Physiology, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, Gebouw U, B-2610 Wilrijk, Belgium; [2]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [3]Embrapa Dairy Cattle Research Center, Juiz de Fora, MG 36038-330 Brazil, Peter.Bols@ua.ac.be

SUMMARY

Transvaginal, ultrasound-guided follicular aspiration (OPU) is the method of choice for to retrieve immature oocytes from highly valuable living donor cows for *in vitro* embryo production. This paper is an overview on the most important technical and biological factors that determine the success rate of ultrasound-guided transvaginal oocyte retrieval in the cow. In the introduction, we comment on general techniques of oocyte retrieval while briefly offering possible criteria for follicle and oocyte selection. In the second part, factors that influence the quality and quantity of the oocytes retrieved are discussed. Firstly, there is a group of technical factors such as aspiration procedure, needle type, and the influence of the aspiration vacuum on cumulus oocyte complex morphology. Secondly, there is a huge group of biological influences, e.g. hormonal stimulation prior to follicle puncture, timing of the procedure relative to the estrous cycle, age, breed, and physiological condition of the donor. Subsequently, the use of OPU in combination with *in vitro* embryo production for the treatment of bovine sterility patients is discussed, as a way to obtain additional offspring from donors with impaired fertility. Reports on donor animal health and reproductive performance following repeated OPU are commented upon. Finally, future research possibilities related to the OPU technique are discussed. Additional gain is expected from the development of better ultrasound equipment, permitting optimal visualization of the smallest follicles. In addition, OPU equipment will be used for related purposes, such as repeated retrieval of ovarian biopsies, and the injection of local ovarian mediators, which will permit to study local paracrine hormonal effects, and their influence on oocyte quality.

7

INTRODUCTION

For several decades now, puncture of bovine (immature) ovarian follicles has been used to retrieve oocytes for *in vitro* production of embryo's (IVP). Several comprehensive updates reviewing IVP and embryo transfer in domestic animals indicate that the availability of 'good' quality oocytes is the most important pre-requisite for success (Hasler, 1998; Galli *et al*., 2001; Merton *et al*., 2003). Puncture of immature follicles on ovaries in living donors clearly opened new perspectives in assisted reproduction

programs because additional gametes become available for *in vitro* embryo production (IVP) over a long time period, which is not the case once the donor animal is slaughtered. In addition, oocyte retrieval during life permits endocrinological modulation of the donor's ovarian activity prior to oocyte retrieval, to influence the quantity and quality of the retrieved cumulus oocyte complexes (COCs). A few basic differences exist between post-mortem and *in vivo* oocyte retrieval. Firstly, transrectal manipulation of the ovary is possible, or even necessary, during oocyte retrieval in the living donor, to achieve follicle visualization through laparoscopy or ultrasound imaging. In contrast, when follicles on slaughterhouse ovaries are punctured, a specific follicle can be punctured under direct visual control. Secondly, different mechanical forces play a role while puncturing follicles *'in vitro'*, compared to '*in vivo'* follicular aspiration with an adjustable aspiration vacuum pressure (Hashimoto *et al.*, 1999a). Different methods have been used to repeatedly collect oocytes in living donor cows, such as puncturing follicles during laparoscopy (Schellander *et al.*, 1989), which results in high recovery rates, but has the disadvantage of being relatively invasive and laborious. Callesen *et al.* (1987) were the first to use ultrasonography to collect oocytes in living cattle, using a transcutaneous approach while a technique of transvaginal laparoscopy was described by Reichenbach *et al.* (1994). Finally, Pieterse *et al*. (1988) modified a transvaginal ovum pick-up technique, originally developed for use in human reproduction (Dellenbach *et al.*, 1984), for use in cattle. This way, a low invasive method with a high repeatability (Pieterse *et al.*, 1991a) for oocyte retrieval in living donor cows was available. Becker and colleagues (1996) compared transvaginal oocyte retrieval under ultrasound control, or in combination with endoscopic instruments. They concluded that the use of ultrasound equipment resulted in better quality cumulus oocyte complexes, probably because of the greater extent of COC damage caused by the endoscopic aspiration system, which was confirmed by Santl *et al.* (1998).

The OPU technique was adapted for use in living donors to repeatedly retrieve oocytes from selected heifers and cows of high genetic merit (Kruip *et al.*, 1994), to breed large numbers of calves with known production traits, and to shorten the generational interval in cattle breeding programs. Pieterse *et al.* (1988) stated that the ultimate aim was to produce more embryos and pregnancies per donor cow than through superovulation and classic embryo transfer (ET) programs. The outcome of OPU, in terms of numbers and quality of retrieved cumulus oocyte complexes (COCs), is influenced by technical as well as biological factors (Bols, 1997; De Roover *et al*., 2005), which will both be discussed in detail. For practical reasons, 'ultrasound-guided transvaginal oocyte retrieval' or 'ovum pick-up' will be abbreviated to 'OPU' throughout the rest of the paper.

OOCYTE RETRIEVAL: WHICH FOLLICLES TO PUNCTURE?

An insight into the relation between follicular diameter and the quality of the enclosed COC seems of vital importance for follicle and oocyte selection. Price *et al.*, 1995 used ultrasound technology to follow the sequential development of a particular follicle in time. They observed a direct correlation between diameters of dissected follicles and diameters observed by ultrasonography, the latter being

consistently 2-3 mm smaller. Each follicle can be considered as a micro-environment for the enclosed oocyte (for recent review Gilchrist et al., 2004). Growth of the dominant follicle is associated with an increase in the concentration of estradiol-17ß in the follicular fluid, which therefore becomes estradiol dominated (Assey et al., 1994). Subordinate follicles have a lower estradiol-17ß/progesterone ratio or were progesterone dominated. Price et al. (1995) noted that estradiol-17ß concentrations were significantly lower in regressing and histologically atretic follicles, compared to non-atretic ones. Although Arlotto *et al.*, (1995) reported oocyte growth in all bovine follicular sizes studied, Fair and co-workers (1995) demonstrated only a small positive correlation between oocyte diameter and follicle size. Apparently, the increase in oocyte diameter leveled off at about 120 µm, as follicle size increased beyond 3 mm, while full meiotic competence was already achieved at a 110 µm. Lonergan *et al.* (1994) obtained more first quality COCs (with many layers of cumulus cells) and a higher number of blastocysts with oocytes from follicles with a diameter > 6 mm. This was confirmed by others (Pavlok *et al.*, 1992; Blondin and Sirard, 1995) who reported poor development of oocytes originating from follicles with a diameter < 2-3 mm. However, when OPU is performed in a commercial embryo production program, usually all follicles will be punctured to maximize oocyte yields.

The method of retrieval clearly has an impact on the cumulus oocyte complex (COC) morphology and subsequent developmental capacity *in vitro* (Bols *et al*., 1996a, 1997). The importance of an intact cumulus cell investment for oocyte maturation and *in vitro* development can not be underestimated (Konishi *et al.*, 1996). Naked oocytes had a significantly lower rate of fertilization, compared to COCs with a complete dense cumulus, giving the highest cleavage rates (Shioya *et al.*, 1988). Immature bovine oocytes are divided into different quality categories, based upon light microscopic evaluation of the compactness of the cumulus cells and transparency of the cytoplasm (de Loos *et al.*, 1989; Hazeleger *et al.*, 1995). Best quality oocytes have a homogeneous ooplasm and are embedded in a compact, multi-layered cumulus investment. The less compact the cumulus cells and the more irregular the cytoplasm, the lower the quality. Close contact between cumulus cells and the ooplasm is established through cumulus cell process endings (CCPEs). In the best quality oocytes, these CCPEs penetrate the cortex and make gap junctions with the oolemma (de Loos *et al*. 1991), whereas this is less or even not the case in lower quality oocytes. Blondin and Sirard (1995) showed that oocytes with signs of beginning expansion in the outer cumulus cell layers and a slightly granulated ooplasm developed past the 16-cell stage significantly more than others.

Unfortunately, not a lot of progress is made when it comes to other, non invasive evaluation criteria for oocyte quality. Oocyte quality evaluation techniques are nearly always invasive, so that oocytes are lost for further *in vitro* culture, following quality assessment. This way, possible conclusions always have to be extrapolated to other non-tested oocytes which are allowed to develop *in vitro* without disturbances. However, hopefull results were obtained recently using the brilliant cresyl blue stain to select more competent prepuberal goat (Rodriguez-Gonzalez et al., 2002) or bovine (Alm et al., 2005) oocytes.

TECHNICAL FACTORS INFLUENCING OPU RESULTS

The OPU success rate is partly measured by the recovery rate (RR = number of oocytes per 100 follicles punctured), which is, amongst others, influenced by follicle visualisation (Bols *et al*., 2004a), needle diameter and aspiration vacuum level (Bols *et al*., 1996, 1997), and operator experience (Scott *et al.,* 1994). As a result, RRs vary between 7% (Scott *et al.,* 1994) and 69.6% (Looney *et al.,* 1994), for different research teams. Recent advances in ultrasound technology have increased image resolution and quality of ovary visualization (Hashimoto et al., 1999). In addition, other ultrasound transducers were tested recently for the use in transvaginal OPU. A linear array transducer (Esaote/Pie Medical, Maastricht, the Netherlands), which is a common, routinely used probe for standard transrectal work (Fig 1.), turned out to be suitable for OPU use, when in experienced hands (Bols *et al*., 2004a). Follicle visualisation though, turned out to be better with the multiple angle mechanical probe (Esaote/Pie Medical, Maastricht, the Netherlands, (Fig 2).

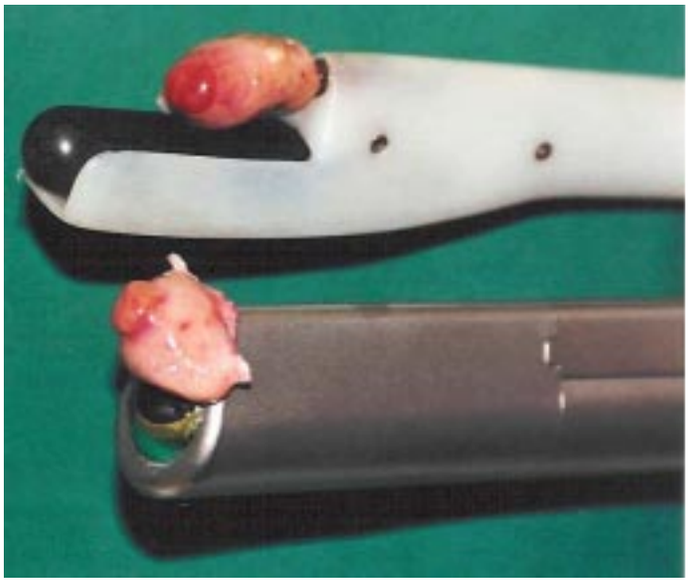

Figure 1. A linear array transducer mounted in an OPU probe holder (top) compared to the multiple angle mechanical probe (bottom).

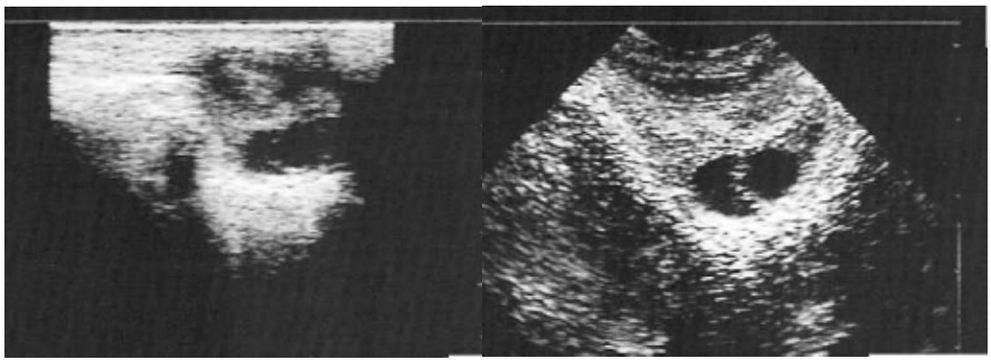

Figure 2. Comparison of ultrasound follicle visualisation of the same ovary between the linear array transducer (left) and the multiple angle mechanical transducer (right).

As reported earlier on, a sharp needle is essential for successful OPU (Scott *et al.*, 1994). Over the years, different needle diameters and aspiration pressures were used in bovine OPU experiments (Table 1), which makes it difficult to compare recovery rates. An important disadvantage of the long non-disposable needles is that they blunt quickly and need resharpening regularly, while they never regain their initial sharpness (Simon *et al.*, 1993). Therefore, we adapted the OPU system to the use of cheap hypodermic injection needles (Bols *et al*., 1995) by combining a multiple angle mechanical sector probe with a unilateral scanning area, and a new disposable needle guidance system.

Table 1. Different aspiration vacuum levels and needle diameters.

Author	Aspiration vacuum	Needle diameter
Pieterse *et al*., 1988	30-40 ml water/ min	0.8-1.0 mm ID[a]
van der Schans *et al.,* 1991	40 mm Hg	18-g
Gibbons *et al*., 1994	75 mm Hg	17-g
Kruip *et al.,* 1994	40-50 mm Hg	0.9 mm ID[a]
Looney *et al.,* 1994	22 ml water/min	17-g
Scott *et al.,* 1994	26 ml water/min	17-18-g
Vos *et al*., 1994	4,4 ml water/min	0.6 mm ID[a]
Bols *et al*., 1995	36 ml water/min	19-g
Bungartz *et al.,* 1995	10 ml water/min	18-g
Stubbings *et al*., 1995	not mentioned	18-g
Becker *et al*., 1996	22,4 ml water/min	0.8 mm ID[a]
Garcia and Salaheddine, 1998	25 ml water/min	18-g
Santl *et al*., 1998	16-20 ml water/min	17-g
Hashimoto *et al*., 1999a	40 to 160 mm Hg	18 and 21-g
Hashimoto *et al*., 1999b	30 ml water/min	18-g
Imai *et al*., 2000	not mentioned	17-g
Goodhand *et al*., 1999, 2000	70-80 mm Hg	20-g
Ward *et al*., 2000	30-90 mm Hg	19-g
Galli *et al*., 2001	20-25 ml water/min	19-g
Petyim *et al*., 2001	12-15 ml water/min	20-g
Seneda *et al*., 2001	65-70 mm Hg	19-g
Argov *et al.,* 2004	25 ml/min	18-g
Hendriksen *et al*., 2004	not mentioned	18-g

[a]ID: internal needle diameter

In addition, the exact aspiration pressure at the top of the needle depends on the construction of the aspiration device, the length and diameter of the tubing system, as well as the needle diameter. To make comparisons possible, the aspiration vacuum should better be expressed as the amount of fluid (in ml) aspirated per minute, rather than in mm Hg. Given the importance of an intact cumulus cell investment for oocyte maturation and future developmental capacity, the damage to the COC, caused by the aspiration procedure, was assessed for disposable needles. The highest RR were obtained with the widest needle (18-g), regardless of the aspiration vacuum pressure, while more oocytes were recovered with a stronger aspiration vacuum (Bols *et al*., 1996). However, more intact COCs were recovered at the lowest

aspiration vacuum (aspiration flow rate of ± 36 ml fluid/ minute), with an average of 75%. The recovery percentage of intact COCs decreased progressively as the aspiration vacuum increased, which was associated with a consistent increase in the number of naked oocytes, as later confirmed by Ward *et al.* (2000). The net effect of the aspiration procedure on COCs, was assessed by re-aspiration of previously scored compact cumulus oocyte complexes (CCOCs) by the disposable needle guidance system *in vitro*. This resulted in a re-collection of 80% or, in other words, one out of five COCs was lost in the aspiration system (Bols *et al.,* 1997). A more complex route for the oocyte to follow during aspiration is inherent to a disposable needle guidance system. Between 80 and 90% of the recovered CCOCs were still surrounded by a compact cumulus investment following aspiration, while the remaining 10 to 20% are microscopically damaged by the OPU procedure, in a way that cumulus cells are totally or partially stripped off, impairing the oocyte's *in vitro* developmental potential (Zhang *et al,* 1995).

BIOLOGICAL FACTORS INFLUENCING OPU RESULTS

Frequency and timing of follicular puncture

The OPU technique has the advantage of being highly repeatable. Pieterse *et al.* (1991) punctured follicles during different estrous cycle stages, over a three month period. Vos and co-workers (1994) could retrieve 5 times more COCs, 22 hrs following the LH peak, compared to shortly prior to the LH surge. The proportion of good quality oocytes was higher for cows punctured on day 5, compared to day 8 of the cycle while the dominant follicle apparently reduces the developmental competence of oocytes from subordinate follicles, even at a relatively late stage of dominance (Hendriksen *et al.,* 2004). Van der Schans *et al.* (1991) punctured a larger number of follicles (46.0 vs 25.3) and harvested more COCs (18.8 vs 10.8) in stimulated cows, when the ovaries were punctured twice, instead of once per week. The collection frequency affected neither the number of follicles aspirated nor the number of COCs collected per session, as later confirmed by Garcia and Salaheddine (1998), who concluded that follicle aspiration appeared to induce and synchronize follicular waves. Most researchers agree that a twice-weekly oocyte collection scheme has a positive effect on the number of follicles available for puncture (Simon *et al.,* 1993), and the number of cultured blastocysts (Gibbons *et al.,* 1994). It can be assumed that the dominant follicle is ablated during each session when a cow is punctured twice a week, thereby stimulating an additional wave of smaller follicles to grow (Bergfelt *et al.,* 1994). However, others report optimal results with an interval of 7 days between subsequent OPU sessions (Imai *et al.,* 2000).

Physiological and body condition of the donor

OPU is generally performed in cattle breeding programs involving healthy heifers with excellent traits. However, pregnancy does not exclude OPU, since oocytes can be retrieved within the first 3 months of pregnancy, even following FSH treatment (Meintjes *et al.,* 1995), although this will not

always increase the number of oocytes retrieved (Bungartz *et al.,* 1995). Dominguez (1995) counted fewer medium and large follicles in pregnant, compared to cyclic cows. Argov and colleagues (2004) saw an increase in the number of oocytes obtained, with the number of aspiration sessions, in cows in early lactation. Undernutrition had a negative influence on the developmental competence of oocytes *in vitro,* illustrated by a decreasing number of blastocysts with a decreasing body condition score (Lopez Ruiz *et al*., 1996) and an increasing proportion of good quality oocytes with increasing body condition score (Dominguez, 1995). Leroy and colleagues (2004) demonstrated that typical metabolic alterations, found in serum of high yielding dairy cows shortly post partum, are reflected in the follicular fluid of the dominant follicle, possibly affecting the oocyte's developmental competence.

Breed and age of the donor.

European breeds apparently had significantly more large follicles, compared to Zebu or crossbred cows (Dominguez, 1995), while no differences in the proportion of normal oocytes could be detected. De Armas *et al*. (1994) reported that the Holstein breed gave the highest number of COCs per ovary, but the lowest cleavage rates, and crossbred animals (Zebu x Holstein) gave the highest proportion of blastocysts. Increased use of OPU-IVF in tropical areas will definitely generate more data in the near future. The use of OPU in young donors is limited because of the smaller dimensions of the pelvis. Holstein Friesian heifers can be subjected to OPU, starting at the age of 6-8 months, depending on the dimensions of the intravaginal handle and transducer used (Bols *et al*., 1999). Calves can be punctured, but require a different approach of the ovaries (Brogliatti *et al*., 1995). One of the major problems with prepuberal donors is the impaired *in vitro* developmental capacity of the oocytes (Taneja *et al*., 2000). Although large numbers of follicles can be generated using exogenous gonadotrophins, oocytes seem to lack developmental competence. Large variations in oocyte and blastocyst yield can exist between different donor animal populations. We collected on average 3.1 oocytes per session using non stimulated Belgian Blue donors with impaired fertility, compared to 6.1 oocytes with healthy Holstein Friesian heifers. The corresponding average numbers of blastocysts per session were 0.5 and 1.5 respectively (Bols *et al*., 1996b, 1998).

The role of hormonal stimulation prior to OPU.

Although Pieterse *et al.* (1991b) stated that repeated OPU is possible in non-stimulated cows, or in all situations were follicles larger than 2 mm are present (Kruip *et al*, 1994), low follicular activity needs to be stimulated in some donors, mostly by using FSH-LH combinations or pregnant mare serum gonadotrophin (PMSG, eCG). While these are well known hormones in ET programs, modifications in dose and timing of treatment are necessary, because the final aim of stimulation prior to OPU is to generate additional follicles, instead of initiating ovulation. Pieterse *et al.* (1988) found the highest recovery rates in PMSG treated donors, while their ovaries were larger and produced more follicles compared to non-stimulated animals. Later work however (Pieterse *et al*., 1992) showed a higher total number of aspirated follicles per cycle in stimulated sessions, but an opposite effect on RR, which was

lower in stimulated vs non-stimulated donors. Looney *et al*. (1994) reported an increase in the number of aspirated follicles and retrieved oocytes following FSH treatment, wich was later confirmed by Meintjens *et al.* (1995) in pregnant donors. Positive effects of FSH, on the number of follicles with a diameter > 6 mm (Lonergan *et al.*, 1994) and the number of viable blastocysts (Van Soom *et al.*, 1995; Goodhand *et al*., 2000) were reported. However, the increase in the number of follicles is often inconsistent, as demonstrated by Paul *et al.* (1995) who found a significant rise in RR and number of morulae in FSH-treated animals, depending on cycle stage. Stubbings and Walton (1995) found no differences in the mean number of follicles available for puncture each week between non stimulated cows, punctured twice a week, and FSH stimulated cows, punctured only once. Subtle changes in FSH dosage influenced the sizes, but not the numbers of follicles, which was merely influenced by the individual donor and OPU session (De Roover *et al.*, 2005a). Detailed analysis of the number of follicles and oocytes collected following stimulated OPU sessions revealed distinct reaction patterns to FSH stimulation. Low, medium and high responders could be characterised on the basis of differences in the number of follicles developed, COCs retrieved, blastocysts cultured and recovery rates (De Roover et al., 2005b) It should be noted that FSH treatments can induce asynchrony between maturation of the oocyte and its surrounding follicle (de Loos *et al.*, 1991) or between nuclear and cytoplasmic maturation (Bousquet *et al.*, 1995), resulting in reduced developmental rates.

Inconsistent and unpredictable results with traditional stimulation protocols, lead researchers to investigate alternative stimulation therapies. Because of the positive influence bovine somatotropin (BST) has on follicular growth (De La Sota *et al*., 1993; Gong *et al.*, 1993), we investigated the effect of a long-term BST treatment on the follicular population prior to OPU (Bols *et al*., 1998). Although results showed a significant increase in the total number of follicles in the rBST-treated group, no increase in the number of retrieved oocytes could be detected. This might have been caused by a BST-mediated cumulus cell expansion (Izadyar *et al*., 1996), by which the COC got trapped in the follicle or the aspiration system at the moment of retrieval. Murakami *et al*. (2003) demonstrated a positive effect from the combination of FSH and BST on the number of punctured follicles and viable oocytes retrieved. Most likely, hormonal stimulation and the OPU puncture frequency are interrelated in determining the final embryo yield. De Ruigh *et al*. (2000) concluded that FSH treatment, prior to OPU once every two weeks, resulted in significantly more COCs and more embryos produced *in vitro*, expressed per OPU session, as compared to a twice per week non-stimulated OPU scheme. However, the total embryo production over a two week period turned out to be higher with the twice weekly scheme (4 non stimulated sessions in 2 weeks) compared to one FSH stimulated OPU session every two weeks. Goodhand *et al.* (1999) concluded from their experiments that FSH treatment of bovine donors aspirated once a week, produced a similar number of transferable embryos per donor week as did aspiration twice a week, without FSH treatment.

THE USE OF OPU IN THE TREATMENT OF BOVINE STERILITY PATIENTS

Compared to ET, where a cow can be flushed 3 to 4 times a year, with an average yield of 5 embryos per flush, OPU can be performed twice a week. In healthy donor cows, 2 embryos per donor

per week can be produced, equaling 4 to 5 times the average yield from classic ET (Kruip *et al*., 1994). Additional advantages are the possibility to stimulate ovarian activity prior to OPU, and to change the sire-dam combination *in vitro* with oocytes from the same OPU session/ donor, which can hasten the selection proces. On the other hand, OPU/IVF can be used to generate additional offspring from valuable cows that no longer respond to embryo flushing treatments. The first OPU calves in Belgium were born in 1995, following oocyte retrieval in Belgian Blue donors with impaired fertility (Bols *et al*., 1996). Following the transfer of 56 IVP embryos, 12 pregnancies were obtained, leading to at least one calf for 7 out of 12 genetically high valuable donors. Given the narrow genetic basis of the breed, some breeders were convinced that this could make the difference. Looney and co-workers (1994) reported on OPU in 200, mostly beef cattle donors, of which 50% had a history of good embryo production. An average of 6.3 oocytes per session were retrieved, and 16.4% developed to the blastocyst stage, while transfer of 813 embryos resulted in 325 pregnancies (40%). Hasler *et al*. (1995) carried out similar work on 155 infertile dairy cows. An average of 4.9 oocytes per session were retrieved, with 4.1 oocytes being suitable for IVF. Following transfer of 2268 fresh embryos, 1220 pregnancies (53.8%) were obtained. Large data sets illustrate that *in vitro* embryo production, following transvaginal oocyte retrieval, has reached the routine stage with 5 and up to 8 embryos per session (Merton *et al*., 2003). However, the ultimate success of any assisted reproduction technique is measured by the number of calves produced. Therefore, there is also a need for a large stock well synchronized, healthy, recipient cows into which fresh embryos can be transferred. To increase the survival rates of frozen-thawed IVF embryos, optimization of cryopreservation protocols will remain an important issue in future research.

DONOR ANIMAL HEALTH AND (REPEATED) OPU

Reports on the impact of the OPU procedure on donor animal health and future reproductive performance are scarce. Pieterse *et al*. (1991) did not find any adhesions following OPU, and the procedure did not seem to affect the donor's future fertility. More recently, dairy heifers were closely monitored during two periods of 4-5 weeks, while enrolled in a twice-weekly OPU schedule (Petyim *et al.*, 2000). They occasionally showed oestrus signs and corpus luteum-like structures developed from punctured follicles, which concurred with our findings that, based on progesterone profiles, repeated OPU appeared to induce a certain degree of acyclicity (Bols *et al*., 1998). At the end of their first OPU period, these heifers regained their normal cyclicity. Postmortem findings, following the second period, included a thickening of the ovarian tunica albuginea and a slight hardening of the ovaries. The authors concluded that OPU did not seem to cause major negative effects on ovarian structure, nor on subsequent ovarian function (Petyim *et al*., 2000). Additional research revealed a significant rise in FSH levels on the day following puncture (Petyim *et al*., 2001). Heart rate and cortisol levels increased significantly following restraint and epidural injection, but both parameters returned to normal within 10 minutes after completion of the procedure.

GOALS FOR FUTURE RESEARCH

Although transvaginal ultrasound-guided ovum pick-up now is a well established method for oocyte retrieval, (Bousquet *et al*., 1999, Galli *et al*., 2001), research continues both on the technical and biological aspects. A further increase in the number of oocytes retrieved can be expected, thanks to improvements on follicle visualisation (Singh *et al*., 2003). Routine aspiration of smaller follicles, which proved to be beneficial in increasing oocyte recovery rates (Seneda *et al.,* 2001) will be possible. Additional studies will be conducted to further refine, simplify and economize the technique to make it available on a broader worldwide scale. Recently, another transducer was adapted for the use in bovine OPU. The R10 probe (Esaote/ Pie Medical, Maastricht, the Netherlands) is a 7.5 MHz convex array transducer with an 150° scan angle. It was mounted in a custom designed probe holder (Nutricell, Campinas, Brazil) next to a disposable needle guidance system (Figure 3). This R10 probe performed well on follicle visualisation, because of the easy ovary manipulation within the wide scan area and the high resolution image. Preliminary results gave an average of 5.1 ± 3 oocytes in non-stimulated Holstein Friesian donors (Aerts et al., 2004). The device is now being tested for routine use in several large scale set-ups.

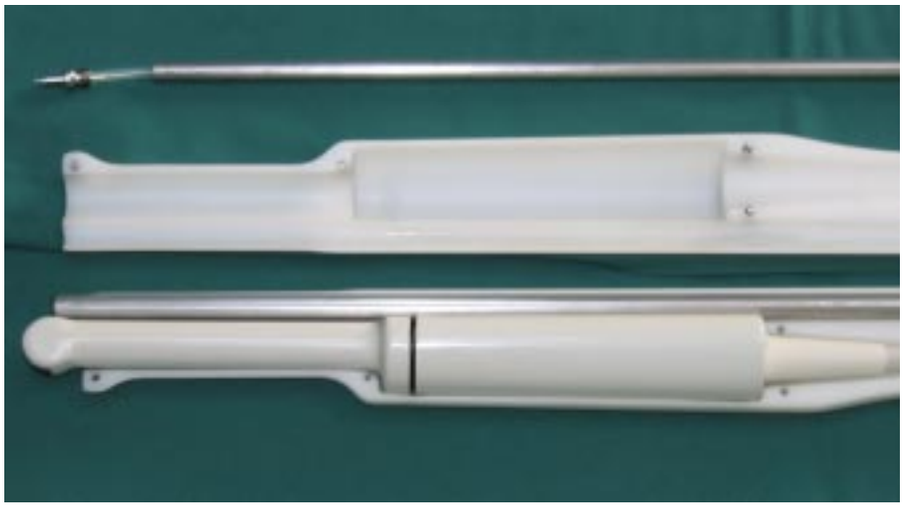

Figure 3. The R10 convex array transducer (Esaote/Pie Medical, Maastricht, the Netherlands) in a custom designed OPU probe holder (Nutricell, Campinas, Brazil)

In addition, OPU equipment is now used for transvaginal injection of substances directly into the ovary, to study and modulate ovarian activity. Oropeza and colleagues (2004) used intra-ovarian injection of Insulin-Like Growth Factor I, in an attempt to overcome developmental deficiencies of prepubertal cattle oocytes, leading to an increase in blastocyst yields. We injected a moderate dose of FSH directly into the ovary to overcome excessive ovarian stimulation. Preliminary results indicate a corresponding moderate ovarian stimulation, with an increase in the number of medium-sized follicles available for puncture (Bols *et al.,* 2004b). Recently, research on follicular dynamics has been redirected towards primordial and preantral follicular populations. Whereas antral follicles can be studied by ultrasound visualization, preantral follicles can only be visualized on stained ovarian tissue sections obtained following slaughter or through laparotomy, which does not allow dynamic follow-up studies. Therefore, we

developed and tested a new method to repeatedly take ovarian biopsies in living donor cattle through ultrasound-guided transvaginal puncture of the ovary (Aerts *et al*., 2005). To our knowledge, only one previous report was published on a similar technique for transvaginal corpus luteum biopsy in cattle (Kot *et al*., 1999). The biopsy technique could be a useful tool for a number of experimental and diagnostic purposes, such as to determine the number of primordial follicles in ovaries of living donors. In addition, repeated ovarian biopsies can be used to study the effects of hormonal treatments '*in vivo*'. Finally, it is generally accepted that primordial follicles constitute the ultimate starting material for *in vitro* culture. It would be very interesting to possess a renewable source of primordial follicles for culture. Our transvaginal biopsy technique presents itself as a minimally invasive, repeatable procedure for collection of primordial and preantral follicles.

GENERAL CONCLUSIONS

Transvaginal ooycte pick-up is a well documented, routinely used procedure for oocyte retrieval in living donor cows, both for research purposes as well as for commercial applications. Its success, which can be measured by the number of good quality, compact cumulus enclosed oocytes retrieved per session, is essentially dependent upon both technical and biological factors. While the former have already been studied intensively, the most progress is expected from controlling the latter. Future experiments will include alternative stimulation protocols to increase the number of follicles available for puncture, and the number of oocytes retrieved. Several well-designed studies have indicated that the impact of OPU on donor animal health and future reproductive life, is of limited proportions. However, considerations of animal health and welfare should always be paramount because good donor health will be the prerequisite for repeated retrieval of numerous, good quality oocytes.

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IN VITRO PRODUCTION OF BOVINE EMBRYOS AN OBSTETRIC OVERVIEW OF PROBLEMS AND CHALLENGES

Prestes, N.C.

Adjunct Professor at the Department of Animal Reproduction and Veterinary Radiology FMVZ – UNESP, Campus of Botucatu, Distrito de Rubião Júnior , S/N, Botucatu – SP Brasil, 18618-000.
nereu@fmvz.unesp.br

INTRODUCTION

Charles Darwin (1890-1882) was a methodical and laborious English naturalist that impressed by South American Fossil characteristics and Galapagos Island species defended the theory of Natural Selection. In 1838, he was introduced to the "Essay on Population" by Malthus that demonstrates a population growth at a geometric proportion until being limited by the food supply, which is characterized by an arithmetic growth.

Regarding the discussed subject, Darwin wrote that "Populations well prepared by continuous observations of animal and plant habits can decrease the fight for existence present in all places. I have observed that under certain circumstances, favorable variations would be conserved, and unfavorable ones would be destroyed, resulting in new species". In 1859, he published the book "The species origin through natural selection, or the preservation of favored breeds by the fight for life" (Storer, Usinger, 1977). Malthus theory was putted down by the species natural selection, where able genetically superior individuals are maintained.

The food production in large scale from small cultivating areas became a reality with the recent application of technologies. The science development in general is responsible for creation of new techniques which will provide conditions for improvement of life quality and environmental conservation.

From the past 50 years, the biotechnology advance has corresponded to the necessities of production and consumption. The animal reproduction area has shown solid advances on semen cryopreservation, artificial insemination, superovulation, embryo transfer, follicle aspiration, *in vitro* fertilization, embryo and semen sexing, transgenesis, cloning and other areas. Reproductive efficiency can be increased by these areas, providing a rapid genetic gain from production animals as a consequence.

Another perspective for advancement has been the manipulation of genetic material by selection of genes associated with specific characteristics, which can be achieved by altering germinative cell lines and providing animals for traditional breeding programs.

The precise genetic manipulation of production animals, specially the bovine specie, has an economic aspect related to the positive modifications resulting from this technology. Some of those consist of growth rate, body condition score, disease resistance and possible creation of bio- reactors for pharmaceutical products leading to a revolutionary change in human therapeutics (Wolf, 2001).

The progress and effort exerted by the scientific community and private organizations for the consolidation and control of biotechnology has been notorious. The multiplication of female and male showing desirable productivity added to their management in adequate conditions became a reality

with the use of efficient techniques.

It is primarily important to mention the role of artificial insemination, in addition to estrus induction and synchronization, which are considered fundamental tools for the multiplication of genetically superior animals.

Starting from the last One Hundred years, the development of biotechnology viewing food production in a high quantity and quality has originated enough knowledge influencing the pattern and longevity of human life.

The most important advances were achieved by gene manipulation and the molecular biology techniques can be with no doubt considered responsible for a revolution of accumulated concepts throughout the years.

However, the ethics and control of products originated from techniques that are not completely established must be present while implementing a new biotechnology, which in turn can prevent the exposure of future generations to an undesirable outcome resulting from its incorrect use (Gonçalves et al., 2002).

The same authors emphasized that the primary objective of cloning is not the creation of copies from the best herd animal, but the improvement of available animals. The best animal from the Fifteens is probably inferior than the average animals from now, in respect of productivity. This shows the power of natural selection and men's induced and guided selection.

THE PROBLEMS AND CHALLENGES

The present subject deserves measured considerations, since statistical and scientific data representing the national herd are lacking in the literature. The observations made by the field service professionals are multi- factorial and they depend on the recipient cow conditions, in respect of nutrition, general herd management, calving monitoring, and geographic factors associated with environment and climate.

Another factor influencing the outcomes is the degree of qualification acquired by the specialized technicians and farm employees.

Professional with no efficient training and knowledge added to a low salary are responsible for maintenance, care and first adds during calving of products with high values in a genetic and economic point of view.

Hasler (1996) reported the abortion frequency in gestations originated from *in vitro* produced (IVP) embryos using TCM- 199 medium, being the loss rate at birth from products originated by IVP reported as 15 %, which must be compared to the 9% loss rate for conventional embryo transfer (E.T) calves. In addition, the hydroallantois condition, once diagnosed has shown a loss rate of 1%.

Those figures represent a significant increase at the abortion frequency if compared to 1 case in 7,500 births/ per natural service.

Birth weight increased has been described and they are known to be 7 Kg higher in calves from IVP than in calves originated by artificial insemination, being similar to findings observed in sheep.

Although not very well quantified, a large amount of recipients have not presented a characteristic

pre-partum phase which can result in losses and interventions followed by C-sections.

Peixer, Dode and Rumpf (2000) described among other findings, some of the numerous factors associated with pregnancy obtained from *in vitro* fertilized embryos, which are represented by high birth weight, prolonged gestation, congenital abnormalities, in addition to increased abortion and perinatal mortality rates.

Farin, Grosier and Farin (2001) reported that a large amount of published information regarding bovine IVP reflects the effect of different protocols and culture medium conditions on embryo morphology and subsequent fetal development. Differences on oocyte sources and maturation, sperm preparations, conditions of fertilization, culture environment, and other factors have a crucial influence on development, sanity and quality of embryos.

A large review from the literature has described the following alterations associated with *in vitro* embryo production systems.

Observed in calves:

- high birth weight
- altered organ development
- congenital malformations, as cerebellar hypoplasia
- altered energy metabolism
- high neonatal mortality

Observed in fetuses:

- increase on non - proportional birth weight
- altered development of muscle fibers
- increase on premature parturition

Observed in placenta:

- increase on hydroallantois
- edema
- reduction at the fetal- maternal contact
- low number of placentomes

Observed in recipients:

- increase on abortion rates
- prolonged gestation
- increase on dystocias and number of cases requiring C- sections

The losses that occur during gestation have been described and the periods of occurrence determined: losses at the embryonic phase (from1 to 42 days) when the organogenesis is finished; fetal phase (from 42 to 280 days), and the neonatal phase (from parturition to 28 days post-partum).

The macro and micro placental alterations added to the progesterone influence in bovine and ovine conceptus are part of the described findings. In addition, numerous factors are known to interfere with the donor cows.

In conclusion, those authors are in agreement with the fact that the exposure to an *in vitro* environment during the first seven days of life can affect embryo morphology and gene expression,

interfering with bovine placenta and fetal development. Those events would be responsible for the large offspring syndrome (LOS) involving biologic mechanisms, which remain unknown.

The knowledge regarding how different components from *in vitro* systems can interfere with placenta and fetal development would lead to a safe method for culture medium, producing embryos, fetuses and calves that would be considered 100% normal.

Histology findings from the necropsy performed on calves originated by IVP are described at Table 1. Those calves died during normal parturition, C-sections, or couple hours after birth, being presented to the Veterinary Teaching Hospital (VTH- UNESP), Campus of Botucatu, Sao Paulo - 2004 and 2005.

The etiology was determined in some cases by the necropsy report, as shown at Table 2. However in a large amount of cases, it was not possible to have a final diagnosis.

Table 1- Necropsy findings from IVP calves (n = 34).

Findings/ Organs	Kidney	Spleen	Heart	Liver	Lungs	S.I.	Thimus	Brain	Cortex
Congestion	25	16	03	17	22	05	12	12	02
Autolysis	01			01		03			
Macrovacuolar Deg.	03		01	08					
Epithelial Deg.	03								
Tubular Deg.	11								
Microdroplet Deg.	06			06					
Macrodroplete Deg.	10			10					
Vacuolar Deg	01		01						
Hydropic Deg				02					
Inflammatory Infiltrate	02		02	04	10	08	02		01
Necrosis	11			02		06			
Edema	03		03		19	01		03	01
Gliosis								10	02
Lymphoid Rarefaction							01		
Colestasis				05					
Hemorrhage	06	02	04	03	07	03			
Enphysema					10				
Atelectasis					11				
Thickness		03		01					
Esteatosis				01					
Retraction		02		01	01	01			
Telangiectasia				01					
NSF	02	05	17		02	06	05	01	

Deg.: degeneration, S.I: small intestine; NSF: no significant findings.
Piagentini, M.; Rocha, N. S.; Prestes, N. C. Partial results - research project in processing (2004-2005).

Table 2 – Final necropsy reports from IVPcalves (n= 13).

Final report	Number of animals
Septic shock from pneumo-enteritis	01
Mononuclear hemorrhagic hepatitis	01
Endotoxic shock from hemorrhagic enteritis	01
Septic shock from bacterial bronchopneumonia	01
Endotoxic shock from bacterial enteritis	01
Septicemia and omphalophlebitis	01
Endotoxemia	01
Septicemia	01
Respiratory insufficiency	03
Endotoxic shock	01
Suppurative pneumonia and hemorrhagic abomisitis	01

Piagentini, M.; Rocha, N. S.; Prestes, N. C. Partial results - research project in processing (2004-2005).

The establishment of *in vitro* culture systems for mammal embryos has contributed to the use of embryo technology at the numerous research areas and at the industry; in addition to its clinical applications (Farin, Farin and Piedrahita, 2004). Studies involving bovine, ovine and rats have concluded that embryos produced by those systems present a different morphology and a distinct potential for development when compared to embryos produced *in vivo*. The article emphasizes the gene expression role on the development of a large neonate (LOS).

Macro and microscopic placental alterations with reduced placentome numbers followed by their enlargement of thickness and diameter have been described by Miglino (2004) in animal cloning and placentation. Abnormal fetuses, presenting enlarged liver with swollen edges and dermal hemorrhage are some of the reported post-mortem findings. The same author mentioned that the hydroallantois condition can be observed at the third trimester during the bovine gestation, which results from an increased production of a plasmatic maternal glycoprotein (PSP 60) by the binuclear throphoblastic cells migrating to the uterine ephitelium. In addition, placentome edema and hemorrhage of are suggestive findings of compromised gestation, as described by Pereira et al (2004).

Few studies describe the mortality rates of embryos from beef cattle and dairy heifers during either a late embryonic phase or early fetal phase. The low incidence of loss is represented by ≤ 10%, with the exception of dairy heifers and beef cattle when used as recipients for *in vitro* produced embryos (Sartori, 2004).

FINAL CONSIDERATIONS

A modern available reproductive biotechnology has been introduced in this country at an explosive commercial scale. The complete knowledge and control of this growing event deserves attention from technicians, researchers, private organizations and cattle raising farmers. Those individuals must provide resources and give efforts to improve the process, and consequently bring the technique to perfection. Once this role has been assumed by the society, it will be possible to correct the actual distortions through the access of physiological and chemical mechanisms. Capacitate employees and control of

basic requirements to perform those biotechniques can bring benefits for the veterinarian class, such as the employment growth and the consolidation of procedures in a safe manner with total credibility.

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WORK SHOPS

Bicudo S.D., Azevedo H.C., Silva Maia M.S., Sousa D.B. & Rodello L. 2005. **Peculiar aspects in sheep artificial insemination.** *Acta Scientiae Veterinariae*. 33 (Suppl 1): 127-130.

PECULIAR ASPECTS IN SHEEP ARTIFICIAL INSEMINATION

Bicudo, S.D.*; Azevedo, H.C.; Silva Maia, M.S.; Sousa, D.B.; Rodello, L.

DRARV - FMVZ – UNESP Botucatu, *sony@fmvz.unesp.br

Reproductive characteristics of ovine species

The ovine species is characterized by presenting a very short generation interval. It is due to the fact that the puberty can be started even before four months old in some breeds. The pregnancy lasts five months and the puerperium is just completed between 35 and 60 days. Lambs of meat breeds can reach the target slaughter weight between 60 and 90 days. Furthermore, the ewe estrus cycle is characterized by a 16 days-interval. The estrus duration is about 30 hours and generally the ovulation occurs in the last ten hours. Although the reproductive seasonality is an important characteristic of the ovine species, it is more significant in wool breeds, principally those specialized in meat production and practically absent in hair breeds. No matter the specialization or breed sheep, in the south hemisphere the most favorable reproduction period is between march and may which corresponds to the autumn season.

The ram semen

Particularly, the ram ejaculation is marked by a volume around 1 to 2 mL and also by a total spermatozoa number varying between two and six billion. Usually, healthy rams present a high spermatic motility: 80% or more of cells with progressive movement. The characteristics of kinetics and morphology spermatic in the ovine species are similar to the other ruminants. For biotechnical applications the semen is easily obtained using the artificial vagina and it does not require great efforts to train the rams to respond to this collection method.

Artificial Insemination Modalities

Both the adoption and viability of A.I technique demands a minimum module of herd size to obtain an appropriate economical refund. In all the cases, it should be questioned if the natural breeding (free, guided or controlled) is the best option that attends both the economical interests and the genetic improvement program to be implemented. It should be considered that A.I demands minimum requirements to the breeding management intensification - minimum conditions should be given to achieve the expected results. Fundamentally, the choice of insemination technique depends on its adaptation to the technological level of the herd.

The use of fresh and cold semen requires more work when compared to natural breeding: estrus

detection; semen collection, evaluation and manipulation; and insemination that requests the contention of each ewe. The refrigeration allows the use of the semen until 24 hours after its collection with excellent fertility results. The semen transport might be possible if adapted isothermal devices were used.

Extenders are indispensable to semen refrigeration and preferentially the dilution rate should be 1:1 to 1:2 (v:v). Higher dilutions generally result in low final density of the mixture that implies in a higher semen reflux risk after superficial cervical inseminations. Usually, the insemination dose should contain between 150 and 200 million of viable spermatozoon allowing the insemination around 15 to 30 ewes with just one ejaculate. The main difference of fresh and refrigerated semen use is in the higher technical knowledge and rigidity demanded when semen is submitted to cold.

The modalities of A.I. using fresh or refrigerated semen have more chances to be usual when compared with frozen semen: use less sophisticated techniques of semen deposition in the female genital, request less expensive equipments and demand less accuracy at the insemination moment. It should be considered that the usage of frozen semen allows a higher selection pressure and consequently a higher impact on the programs of genetic improvement. This advantage overcomes the technical restrictions depending on the herd technological level.

Ram semen cryopreservation

The principles and techniques used in ram semen cryopreservation are similar to those used in other domestic ruminants. However, each species has its own seminal composition; spermatic membrane characteristics and sensibility to the cryopreservation process. It is clear that some necessary modifications should be done to attend these particularities. In general it was found higher damaged effects on the ram spermatozoon submitted to cryopreservation. These damages are characterized by changes of the respiratory activity, cryo-capacitation, spermatic-oviduct interaction mechanism, and by a hypothetical increase of embryo mortality. These effects request intra-uterine deposition of the cryopreserved spermatozoon next to the onset of ovulation. Critical points deserve the attention of countless research groups around the world that seek not only the increment of A.I. results but also practical techniques of semen deposition in the ewe genital as an alternative to the laparoscopic insemination.

Insemination Techniques

When compared to the domestic females of the ruminant species the ewe presents small cervical openings with rings not concentrically aligned and do not dilate during estrus. Therefore, the anatomy of the ovine cervix precludes the deposition of spermatozoa into the uterus via the cervix by inseminating instrument. There is a notable difference among the sheep breeds related to the cervical overcome feasibility. Several cervical overcome techniques have been proposed, even so the efficiency of the procedures has been variable and mainly the pregnancy rates using frozen semen are fewer to those

verified in laparoscopic insemination.

In general, the inseminations with fresh or cold semen involve superficial cervical technique that uses a speculum with light source permitting both the visualization and recognition of the cervix vaginal opening. It allows the deposition in the first cervix rings of 100 to 150 µL of fresh or diluted semen as described previously. The high spermatic colonization and longevity in the female genital are sufficient and allow a flexi-time insemination, the implementation of estrus detection at nights and only one insemination in the morning.

The frozen semen application requests intrauterine insemination and the globally used technique is the laparoscopy because its satisfactory results are easily repeatable. Several techniques to cervical overcome facilitate the uterine semen deposition. However, the worst results obtained with this technique compared to those by laparoscopy, have made some authors suppose that the cervical manipulation can induce not well-known effects over insemination efficiency. It is speculated that there are some interaction factors between transcervical insemination and the qualitative patterns of semen dose. Although their efficiency in promoting the cervical dilatation had been demonstrated in some scientific literature, some drugs like the prostaglandins of group E and the oxytocin usually are still not adopted in routine works.

The methods of estrus induction and synchronization are essentially associated to the artificial insemination using frozen semen. About available methods we can mention the use of ram-effect and alimentary flushing, but the most efficient is the one that uses exogenous drugs because it allows effective control of the onset of ovulation.

The use of vaginal devices impregnated with natural or synthetic progesterone from12 to 14 days, associated with application of Equine Corionic Gonadotropin (eCG) between 250 and 500 UI at the moment of the removal device, allows great efficiency in the estrus occurrence and in the concentration of ovulation time in the ewes.

The laparoscopic inseminations are accomplished in a systematic way between 55 and 60 hours after the time of vaginal devices removal and eCG application allowing the adoption of an artificial insemination system based on a pre-fixed time (AIFT) used for decades in ovine species.

Final Considerations

The reproduction biotechnology application in the ovine species, mainly those ones related to A.I., allies the management intensification to the species potential precocity and increases the usage of high genetic merit rams promoting the adequacy of its economical value.

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EARLY IDENTIFICATION OF THE FETAL SEX IN SMALL RUMINANTS BY ULTRASONOGRAPHY

Santos, M.H.B.[1]; Moraes, E.P.B.X.[1]; Moura, R.T.D.[1]; Lima, P.F.[1];
Reichenbach, H.-D.[2]; Oliveira, M.A.L.[1]

[1]Postgraduate Program in Veterinary Science/UFRPE [2]Byerische Landeanstalt fur Landwirtschaft/ Germany

Historic and Ultrasonography Characteristics

The use of ultrasonography in the field of human medicine comes from 1940 and in veterinary medicine since 1950, however, only in the late 70th decade, the quality of this technology had a great impulse with the possibility of getting images in real time (Christopher & Merrit, 1998).

The ultrasound imaging in real-time emits sound waves of a high frequency that reflect the tissue interfaces, characterized by the different acoustic impedance displayed on the monitor with different shades of grey color (grey scale). The ultrasonographic images are classified in anechoics (dark and derived from liquid that does not reflect the sound waves); hyperechoics (bright and derived from tissues with a great reflective capacity); hypoechoics (they change in different shades of the grey scale) (Christopher & Merrit, 1998).

The development of ultrasound equipments of type B-Mode (Scan-B) with higher image resolution and the improvement of the transducers, in the last years, provided greater accuracy and speed in pregnancy diagnosis, and quantifying and sexing foetuses in caprines and ovines (Nan et al., 2001; Bicudo, 2003). For the pregnancy diagnosis are used, generally, simple equipments and more accessible economically; however, for foetal sex determination are need images with more detail of the foetal structures, aspects that are better obtained with equipments more sophisticated, what determines greater investiments from the farmer or from the liberal professional (Bürstil et al., 2002).

Pregnancy Diagnosis and Identification of the Foetal Sex

The date of the first visualization of the different characteristics of the conceptus is variable and the most important factors that determine this variation are the transducer frequency and the way of access (Buckrell, 1998; Kähn, 1994).

The main ultrasonographic images that characterize a pregnancy are the presence of intrauterine liquid, visualization of embryonic vesicle, the detection of at least one embryo, visualization of the heartbeats, identification of the amniotic membrane, visualization of the placentoma, differentiation between head and body trunk, identification of the limbs germinative button, the movements of the

conceptus, delimitation of the umbilical cord and the visualization of the ocular globe (Ishwar, 1995; Chalhoub & Ribeiro Filho, 2002). The determination of the foetal sex by ultrasonography is based on the localization and differentiation of the external genitalia (Müller & Wittkowsky, 1986), being the foetal anatomical structure that makes possible this diagnosis called genital tubercle (GT) (Curran et al., 1989; Caughbrough & Castell, 1988). In equines, the diagnosis can be effected between the 55th and the 75th day of pregnancy (Merkt & Moura, 2000) and in bovines, since the 50th day (Curran, 1992; Stroud, 1996), although the exam performed on day 60th provides greater precision (Barros & Visintin, 2001). The accuracy of the technique is near 100%, such in bovines (Gregory et al., 1995; Viana et al, 200) as in equines (Merkt & Moura, 2000).

In small ruminants, the above mentioned identification can contribute to foetuses sex determination after the artificial insemination with sex determined semen (Cran et al., 1997; Johnson, 200; Garner, 2001), after the embryo transfer with predetermined sex (Gutierrez-Adam et al., 1997) or embryos produced *in vitro* by the technique of intracytoplasmic injection (Cat et al., 1996). Among the practical application of this byotechnique, we can emphasize its importance to the animal production for allowing a better planning in acquiring as in commercializing animals from the own herd (Haibel, 1990), making possible to rationalize the production and the profitability. This enhanced management provides a greater concentration of females in dairy herd and of males in meat herds (Santos et al., 2004).

The sex determination happens in the moment of fecundation and affects the development of the indifferentiated gonad. It is believed that the factor of testicular differentiation (TDF), gene localized in the Y chromosome known as SRY, corresponds to the sex determining region of the chromosome Y. In this way, in the case of the XY sexual chromosome, the Y induces to the masculine gonadic sex. If the primordial cells have only XX sexual chromosomes, the indifferentiated gonads develop in ovaries and establish the feminine gonadic sex (Wilkens, 1982).

In the male embryo, under the hormonal influence, the GT undergoes an enhanced growth and takes the cylindrical shape that originates the penis. In the female, due to the lack of testicular androgens and the probable action of the maternal, placental and foetus ovaries steroids, the GT grows little and forms the clitoris (Wilkens, 1982; Schnarr, 1989); the urogenital sinus remains short and when it dilates originates the vaginal vestibule (Michel, 1986). The urogenital swellings remain separated in order to originate the vulva lips, covering the GT situated in the vestibule. With the trunk enlargement, the genital swellings situate cranial to the GT. (Noden & De Lahunta, 1990). In males and females, the two prominences around urethral folds, called scrotal lips will take part in the scrotum and vulva formation (Wilkens, 1982; Schnorr, 1989).

Morphologically, according to Bürstel (2002), the ovine and caprine conceptus, between the 35th and the 40th day of pregnancy is, under the anatomical point of view, sexually undefined and ambivalent, in spite of the ovine embryos on day 25 of gestation already show, according to Schnorr (1989), a discrete elevation between the hind limb buds, indicating a GT formation. Between the 28th and the 34th day, the GT is more prominent and on day 34 is already possible to identify the embryo sex. With the development of the body embryo and the GT migration toward umbilical cord in males and the tail in females, it is obtained, respectively, the differentiation of this organ in penis and clitoris. Consequently, from this period, the distance from the anus to the GT will be greater in the male than in the female.

By means of ultrasonography, the TG in bovine species can be identified as a structure formed by two elongated lobules, with the appearance similar to two oval parallel bars that reflect by an intensive way the ultrasound waves addressed to them (Curran et al., 1989).

In little ruminants, the technique of foetal determination by ultrasonography is already possible around the 40th day. Nevertheless, it is commendable between the 50th and the 58th day, being also performed until the 64th day, but with an inferior diagnostic precision (Bürstel, 2002). In experiments carried out by the group of the Biotechnology Laboratory of the Department of Veterinary Medicine (UFRPE), it was possible to identify the beginning of the migration of the GT on day 37 of gestation in an unique male foetus of Santa Inês breed and at the end of the 46th day, all the foetuses quantified had already being sexed. In caprines from the American Alpine breed, the beginning of the GT migration happened by the 42nd day and only by the 55th day all the foetuses had their sex determined.

Considering that the GT migration in ovines seems to occur in a more premature way than in caprines, it is recommended to determine the ovines foetus sex from the 50th day and the caprines from the 55th day, remaining alert that it would not be performed after the 70th day of pregnancy, because the foetus size makes difficult the visualization of the anatomic structures responsible for the identification of the foetal sex.

The main factors that restrain or make difficult the technique of sex determination are the movements and the foetus unfavourable position, the crossed limbs or the umbilical cord situated between the limbs. Particularly in the females, the tail position could interfere in the GT visualization (Nan et al., 2001). For the diagnosis of males, it can also be taken as a base, the prepuce presence immediately caudal to the umbilical cord and/or the presence of the scrotal pouch, usually with a triangular appearance, between the hind limbs (Bürstell, 2002).

It is also important to comment that multiple gestation is a restraining factor on the efficiency of sex determination technique for being generally impossible to identify the sex of all quantified foetuses in a single scanning. This event compromises the economic viability of the ultrasonographic activity in consequence to the necessity of other scans, without the security that in the next scan the foetus will be suitable positioned in order to allow sexing (Reichenbach et al., 2004).

Final Considerations

It is interesting to emphasize that besides the species and the gestation period, other factors like breed, age, corporal score of the female, equipment quality, operator´s capacity and hability may determine important variations in the ultrasonographic diagnosis accuracy.

In spite of what it has been said, the diffusion of this technology, mainly in field conditions, depends yet on a greater diagnosis efficiency, in order that the cost/benefit allows the activity maintenance. Besides, new studies about the subject are necessary to define, with higher accuracy, the period of GT migration in small ruminants, trying to anticipate the technique of foetal sex determination in the above-mentioned species.

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USE OF LOW DOSES OF EQUINE PITUITARY EXTRACT TO INDUCE MULTIPLE OVULATIONS IN MARES

Farinasso, A.[1]; Branquinho, J.A.[1]; Rumpf, R.[2] ; Alvarenga, M.A.[3]

[1]Convênio Unicórnio – Laboratório de Biotecnologia em Reprodução Eqüina / Laboratório de Biofísica – Universidade de Brasília - Brasilia-DF-Brasil. farinasso@unicornioequino.vet.br
[2]Cenargen – Embrapa-Brasilia-DF-Brasil; [3]Unesp – Botucatu-SP-Brasil

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INTRODUCTION

Differently of bovine, the equine female is refractory to the hormonal treatments using swine FSH . The super ovulation of the equine female is only induced with the Equine Pituitary Extract (EPE) treatment. Furthermore, this procedure can strongly reduce the costs involved in the embryo transfer program, allowing also the possibility to freeze embryos more frequently(Carmo & Alvarenga,2004). The induction of multiple ovulations in mares can also , multiplies the sources of oocytes increases the fertility of mares inseminated with frozen semen and improves the pregnancy rate in mares with low fertility (Squires et al. 2003).

Despite of all presented advantages of EPE the contamination of the crude extract with high content of LH, and the differences in the hormonal answer could interfere in the multiple ovulation induction (Squires, 2003). Firstly, the daily dose of EPE to induce the multiple ovulation was of approximately 1,5 g for each treatment. Currently, this dose was drastically reduced in the values ranging from 25 mg to 50 mg. However, the effects of daily dose < 25 mg it is not still knowned.

EXPERIMENTS AND CLINICAL OBSERVATIONS WITH THE UTILIZATION OF LOW DOSE OF EPE DO INDUCE MULTIPLE OVULATIONS IN MARES

In order to test the efficiency of the EPE in low doses we compared the treatments with daily doses of 2 mg (n:12), and 6 mg (n:13), in the breeding season of 2003/2004, with a control group represented by the previous cycle of each treated female.

In diestrous (6 to 9 days after ovulation), the mares with follicles ≤25 mm were induced to the oestrus (3 x 0.5 ml of lutalyse, PGF2α), with intervals of 12 hs and treated with I.M. EPE , until 1 or more follicles reached 35 diameter mm in the larger axis. The ovulatory rate of the group treated with 2 mg of EPE was compared with control group . The mares were coupled or inseminated 12 to 24 hs after the ovulation induction with 2500 UI of hCG. Embryo flushes were performed within 8 to 9 days after the last ovulation. Only two mares (16.7%) showed multiple ovulations (2/12) with the dose of 2 mg, resulting in ovulatory rate similar to the control group. The daily dose of 6 mg increased (P

<0.001) the ovulation rate for 1.84 ± 0.58, differing of the control group (1.15 ± 0.37). Indeed, it promoted double and triple ovulations in 76.9% of the treated mares. It was obtained in average 1.31 ± 0.75 embryos by cycle, and 0.54 ± 0.52 (P <0.05) for the control group. These data showed that this dose is able to duplicate the chances of embryo recovery in donors mares.

In the breeding season of 2004 / 2005, commercial donors were treated with 4 mg 2 x day, 6 mg 1 x day; and 8 mg 1 x day of EPE. The EPE was produced at Unicornio/Biophysis -Laboratory- UNB . Multiple ovulation's were obtained for different doses, as following: 54% of mares (33/61) for the group treated with 4 mg 2x-/ day; 60% of mares (31/51) for the group with treated 6 mg 1 x/day, and 63,6% of mares(14/22) for the group treated with 8 mg 1 x/day. The embryo recovery rate per cycle for all doses were:0.94 (4 mg 2x/day); 1.0 (6 mg 1 x/day; and 1.0 (8 mg 1 x/day). The multiple ovulations (number of animals with multiple ovullation / number of treated animals) obtained for 8 different Veterinarians that used the EPE produced in our laboratory were: 81% (22/27); 54% (25/47); 39% (12/31); 58% (7/12); 30% (3/10); 40% (4/10); 100% (4/4); and 100% (5/5). In the first year using EPE at our practice we observed that 76,9% (10/13)had multiple ovulations using 6 mg 1 x/day. In the second year we observed 81% (22/27) of mares with double ovulation using 4 mg 2 x/day. We observed that to have better results the treatment must preferentially started between day 6 to day 9 after ovulation in mares that presented two or more follicles with equivalent sizes.

Several donors were treated during 4 to 6 successive cycles. It was observed in a mare 5 double ovulation and 8 embryos in 6 consecutive cycles, in another mare we observed 5 double ovulation and 8 embryos in 5 consecutive cycles, and two other mares with 4 double ovulation and 6 embryos after treatments in 4 consecutive cycles. Showing that the treatment with low dose of EPE was efficient to induce double ovulations and eventually more than double ovulations in cyclic mares. Therefore, this protocol can be used as a successful and no aggressive hormonal treatment in successive cycles.

The EPE was also tested in 4 non -cycling mares presenting flaccid uterus and small follicle population with approximately 5 to 15 mm. It was observed the induction of multiple ovulations in two treated mares (4 follicles) after two daily applications of 12 mg for 8 days.

CLINICAL OBSERVATIONS WITH THE UTILIZATION OF LOW DOSE OF EPE DO INDUCE OVULATIONS IN MARES

The use of low dose of EPE to induce ovulation was also tested time of an endovenous dose of 6 mg was used, in commercial mares with two or more pre- ovulatory follicles. Mares presenting one or two follicles larger than 32 mm and uterine edema, detected by ultrasonografic, were treated with 6 mg of EPE diluted in 1.5 ml NaCl 0.9% and evaluated by ultra-sound after 24, 36 and 48 hs. From 34 mares were 31 ovulated (91%) until 48 hs after EPE treatment . In 11 cycles exhibiting double follicles, all ovulations ocorred until 48 hs after EPE treatment to induce ovulation, and 68% (15)ovulated until 36 hs. However in five mares presenting 3 to 4 follicles were observed partial ovulation after induction with EPE indicating that the dose of 6 mg was insufficient to induce and to synchronize ovulations in mares with more than two follicles.

CONCLUSIONS

Based on the results of our experiments and in our clinical observations we can concluded that the treatment with of extract of pituitary equine using doses of 4 mg 2 x to the day or doses of 6 mg and 8 mg 1 x a day assures at least 1 embryo per flush (cycle). In Brazil, the costs of a embryo flush oscillate between R$ 600,00 to R$ 1.000,00 reais . The guaranty of 1 embryo per flush minimizes the high costs of the negative collections, making the low dose EPE treatment highly desirable from the economical point of view. The use of EPE as ovulation inductor presents advantage to hCG for no production of antibodies.

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Neves Neto J.R. 2005. **Strategies to improve reproductive efficiency in subfertile stallions in embryo transfer programs.** *Acta Scientiae Veterinariae*. 33 (Suppl 1): 139-140.

STRATEGIES TO IMPROVE REPRODUCTIVE EFFICIENCY IN SUBFERTILE STALLIONS IN EMBRYO TRANSFER PROGRAMS

Neves Neto, J.R.

Instituto Veterinário de Reprodução Assistida, Fleury Reprodução Eqüina
nevesneto@uol.com.br

In order to achieve a good reproductive performance, the stallion should have an efficient fertility rate. Fertility can be defined in several manners, as embryo recovery rate, pregnancies after artificial insemination or mating through a breeding season or in an oestrus cycle. There are many factors related to fertility that interfere in stallion's reproductive performance resulting in different subfertility degrees that have particular characteristic symptoms and treatments.

These factors present themselves due to smaller or greater failures and/or frequency related to altered sexual behavior during mating; libido, erection and ejaculation disturbs; spermatic production with its seminal volume, concentration, motility, spermatic morphology, membrane integrity characteristics within other necessary attributes to certify that fertilization occurs and congenital or acquired problems on stallion's reproductive tract are important aspects to evaluate and determine reproductive performance limitations in each one of these animals. A reproductive evaluation is the first step to diagnose possible failures in fertility and determine reproductive management strategies to these stallions that are in some degree, subfertile.

Another important aspect are the stallions that show fertility problems, as spermatic parameters alterations, when submitted to stress, intense mating regimen and/or seminal collection, the same way, normal stallions, or some that show any degree of subfertility, when included in reproduction biotecnhiques as cooled semen stored at 5 and 8º. C, 13 and 18ºC and frozen semen, have low embryo or pregnancy rates. Young and old animals are more sensitive to these situations.

Important strategies to improve reproductive performance in stallions that are subfertile in different degrees are a specific management, diagnosing the problem and instituting measures capable of solving it or minimizing the lowered fertility, as:

- Adequate management to achieve the animal's well being.
- Rationalized frequency of ejaculations on adult stallions, and specially, young and old. In Brazil, Mangalarga and Mangalarga Marchador breed have the majority of animals with seminal characteristics inferior than others, being the most affected during breeding seasons, compromising their reproductive longevity.
- Supplementation with vitamin complexes (Vitamins A, E, D, β–carotin…), oligoelements (Selenium, Zinc, Magnesium…), Amino acids complexes (L-carnitin, Lysine, Thriptofan, Arginine…) and omega 3 and 6, associated or not to hormonal applications (GnRH or hCG) to improve spermatic production.
- Enriched extender's with one or several substances that improve cellular metabolism and spermatic viability (pentoxifylline, L-carnitin, taurin, heterologous seminal plasma, heparin) in

the moment of seminal collection (total or fractioned) and after transport.

- Utilization of enriched extenders, adapting *swim up* technique, with the lower column represented by the semen and superior column with enriched extender, to animals that have on fresh, cooled or frozen semen, low spermatic quality.
- To improve motility and normality characteristics, fresh or cooled semen can be filtrated, through a column of glass spheres, 250 to 500 micrometers in diameter, sustained by a thin glass cotton layer in a 20ml syringe.
- The centrifugation of fresh semen and after transport, with or without enriched extenders addition, can be a good choice to improve embryo recovery and pregnancy rates for those stallions that show low spermatic quality.
- The intra-cornual or tubouterine junction artificial insemination with fresh, cooled and frozen semen, have been of great value, increasing fertility rates for those stallions that have low seminal characteristics under different semen biotechinques.
- Utilization of hormones, ansiolytics, imipramine chloridrate within others, in occurrence of disturbs related to erection and ejaculation.

In conclusion, the veterinary responsible for reproduction must know all different variables that interfere in aspects of the male's reproductive physiology and always be alert to the thin line that separates fertility from subfertility. In the aim to obtain a greater number of embryos and pregnancies, we must take care not to turn the subfertility to an irreversible state or accelerate the path to infertility, despite the different available techniques that help these animals's reproductive performance.

HAS TAI A PROFITABLE BENEFIT COST?

Madureira, E.H. madureh@usp.br; Baruselli, P.S. barusel@usp.br;
Valim Pimentel, J.R. jrvalim@usp.br; Almeida, A.B. alexbar@usp.br

There is in Brazil a long way to get improvement in reproduction efficiency and use of genetic proved bulls in natural matting (NM) or artificial insemination (AI).

One of the main factors that contribute to reduce reproductive performance is the low service rate (SR), that can be explained by two factors: cyclicity of the cows and low efficiency in heat detection.

Timed artificial insemination (TAI), after progesterone or progestogens treatments, has been performed to solve both problems. Firstly, these treatments induce cyclicity in some percentage of anestrous females and become possible AI to get a SR of 100%, without heat detection.

Several protocols have been developed since 1960, and most of them, got improvement in estrous synchronization as well as fertility (BARUSELLI et al, 2004).

Nowadays, there are some doubts about profitability and economic viability of these programs in beef cattle operations.

One experiment was performed to analyse the efficiency of a TAI program in a beef cattle farm working with AI or natural matting (NM).

Five hundred ninety seven Nelore *(Bos taurus indicus)* suckled cows were divided in four groups, according to the body condition.

The group 1 (G1) were kept in NM during 90 days. The second group (G2) was submitted to AI during 45 days followed by 45 days in NM. Group 3 (G3) underwent TAI after a treatment to estrous synchronization using norgestomet, followed by NM for 90 days to complete the breeding season (BS). Also, the group 4 (G4) underwent TAI and was submitted to AI during 45 days and than to NM until the end of BS. The treatment of estrous synchronization was based on a 3 mg norgestomet ear implant and one injection of 3 mg injection (IM) of norgestomet associated to 5 mg of estradiol valerate at D0 (Crestar – Intervet).

At D9, the implants were withdrawn and the animals received a 150 mg de D-cloprostenol (Preloban- Intervet) and a 400 UI shot of eCG (Folligon- Intervet). TAI was performed 54-56 hours after implant removal. The results are shown in Table 1.

The first important result is for those groups submitted to TAI (G3 and G4) which get higher pregnancy rates, with a 8% increase on average at the end of BS when compared with G1 and G2. The interval between the beginning of BS and conception was 22 days shorter to groups bred by TAI. The number of calves produced by AI will be also different among the groups: zero in G1, 35 in G2, 76 in G3 and 94 in G4. Considering Nelore proven bulls, with 10 Kg of Expected Progeny Differences (EPDs) for weaning weight, it can be stated that AI allows for greater genetic improvement than NM. Besides, cows submitted to AI will have 22 additional days open at the beginning of the next BS, so they will have a greater chance to get pregnant and contribute to reduce the between calving interval.

TAI program is no doubt, an important tool to improve reproductive performance in beef and milk production. The results depend on several factors, such as body condition, effects of suckling and

cyclicity, which can not be disregarded.

Several works have been published about post partum period that can be used to review this subject (YAVAS and WALTON, 2000; WILTBANK et al 2002; RHODES et al 2003).

It is important to record that treatments with progesterone or progestin can induce the cyclicity in anestrous cows (GARCIA-WINDER et al 1986) and minimize the sensibility of hypothalamus to negative effects of estradiol (ANDERSON; DAY,1998). This sensibility to estradiol seems increase during the lactating period (ANDERSON; DAY, 1998) and low energy intake (IMAKAWA, et al 1987).

Special attention must be employed to semen quality used in TAI program and improved efficiency in subsequent heat detection or reutilization of bulls.

Table 1. Reproductive efficiency in Nelore cows submitted to different kinds of breeding seasons (BS). Camapuã, 2005.

	TAI	Heat detection rate	AI conception rate	Pregnancy rate (45d BS)	Pregnancy rate (90d BS)	Interval conception and beginning BS (days)
Bull	-	-	-	44,3 [c] (66/149)	83.2[b] (124/149)	46.5±1.9 [c]
AI + Bull	-	39,3 (59/150)	53,0 (35/66)	23,3 [d] (35/150)	85.0 [b] (125/147)	57.3±2.3 [b]
TAI +Bull	50,7 (76/150)	-	-	75,3 [a] (113/150)	92.7 [a] (139/150)	29.3±2.0 [a]
TAI + AI	54,3 (81/148)	25,4 (17/67)	76,5 (13/17)	63,5 [b] (94/148)	91.9 [a] (136/148)	31.1±2.2 [a]

The column with different letters are significantly different ($p<0.05$) (Penteado et al.,2005)

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IMPORTANCE OF SEMEN QUALITY IN FIXED-TIME ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER PROGRAMS

Arruda, R.P.[1]; Celeghini, E.C.C.[1]; Souza, L.W.O.[1]; Nascimento, J.[1]; Andrade, A.F.C.[1]; Raphael, C.F.[1]; Garcia, A.R.[1]

[1]Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP - 13630-090, Pirassununga-SP, Brasil. arrudarp@usp.br

In attempt to improve artificial insemination (AI) and embryo transfer (ET) programs, several works has been conducted based on the hormonal control of estrous cycle of bovine females and employment of fixed-time AI and ET (FTAI and FTET), eliminating the heat detection. Different drugs, protocols and time of insemination are used with some variation in fertility being reported (ARRUDA et al., 1997; BARBUIO et al., 1999; GARCIA et al., 1999; BERTAN et al., 2004; BARROS; ERENO, 2004; BÓ et al., 2004; BARUSELLI, et al., 2004; MADUREIRA et al., 2004).

After the AI, spermatozoa placed at the female genital tract must cross the uterus, pass to oviduct, by uterotubal junction, and interact to oviduct epithelial to fertilize the oocyte (SARTORI, 2004). To consider the sperm cell as qualitatively viable and potentially fertile is necessary that its morphology, metabolic activity and membranes be normal. The presence of intact membranes is a pre-requisite for the ferlitization-related events, as sperm capacitation, zona pellucida binding and penetration, and oolema fusion can occur (YANAGIMACHI, 1994; RODRIGUEZ-MARTINEZ et al., 1997).

When FTAI and FTET systems are adopted, there is the necessity of use of cryopreserved semen from various donors and several semen batches; so the absence of evaluation or even the lack of criteria in the batch assessment could affect the fertility of the whole female lot, resulting in economic losses, in consequence of drugs cost for synchronization, as well as for ovulation induction, besides the expenses with material, semen, and time.

Fertility reduction associated to insemination using frozen semen has been attributed to processes occurred during cryopreservation of semen, causing damages to spermatozoa, that can be ultrastructural, physic, biochemical, and functional, and that lead the sperm membranes to show bilipidic asymmetry alteration (WATSON, 1995). Loss of motility, chromatin and sperm morphology alterations, increase in permeability and decrease of membrane stability, and generation of reactive oxygen species (ROS) can be included to that damages.

The development of techniques of different fluorescent probes in association that can allow the simultaneously assessment of plasmatic and acrosomal membranes integrity and the mithocondrial function in the bull semen (ARRUDA; CELEGHINI, 2003; CELEGHINI, 2005) has being a useful tool to evaluate the effects of the cryopreservation on the bovine semen. Forero-Gonzalez (2004) has verified that only 15% of the spermatozoa remained intact after the cryoprotection process. Similar results were observed by Celeghini (2005), with values ranging from 18% to 28% after cryopreservation using two different cryoprotectants.

Even more, the spermatozoa, like any other cell, produce ROS from the cell metabolism. The process of lipidic peroxidation initiates in presence of ROS, which in contact with the docosahexaenoic

acid in the sperm membrane, withdraw a hydrogen from an double bond, transforming it to free radical, and by its turn will act on other docosahexaenoic acid. This process triggers off the peroxidation cascade, causing plasmatic membrane lesions, with fluidicity loss and the reduction in the capacity of regulation of the intracellular ion concentration involved in the control of sperm movement and, finally, loss of the fertilization capability (AITKEN; KRAUSZ, 2001; MARQUES et al., 2002). The free radicals generation also can reach the DNA at the spermatic nucleus promoting fragmentation. This fragmentation is frequently observed in spermatozoa of infertile individuals and there are strong evidences that these free radical-mediated damages be induced by oxidative stress (JANUSKAUSKAS et al., 2003).]

The usual laboratory evaluations performed to estimate the potential of fertility of a semen batch are: sperm motility (%); mass movement (1-5); sperm concentration (millions/dose); sperm abnormalities (%); and thermoresistance tests (quick and slow). Those evaluations has been, since the 1980 decade, based on the techniques and minimum standards suggested by the Colégio Brasileiro de Reprodução Animal (CBRA, 1998) for cryopreserved bovine semen assessment (ARRUDA et al., 1992). The sperm motility is often estimated by a subjective way, being analyzed on the optic microscope, with a drop of semen between slide and coverslip, and the estimation is visually taken. The sperm abnormalities are evaluated through stained smears or humid chamber, technique that depends on the technician ability. Although, studies has reported that this type of analysis is imprecise even if carried out by experts. That low precision derives in part from the subjective nature of the uses tests, the variability between technicians and differences in the implementation of the evaluation standards (ARRUDA, 2000).

In attempt to minimize imprecision, a diversity of biotechniques has been developed to seminal assessment, as Computer-Assisted Sperm Analysis (CASA), fluorescent probes to evaluate sperm structures by optical fluorescence microscopy or flow cytometry systems, sperm sexing techniques, seminal plasma proteins determination, and quantification of ROS production.

With the purpose to obtain a technique that provide higher repeatability for both sperm motility and morphology, several systems that use computed analysis of images has been developed and employed. Computer softwares for sperm assessment ca be more objective and increase the repeatability to the evaluation than the human ability to identify patterns of motility and sperm normality (ARRUDA et al., 2003). The power of analysis of this kind of test is given by the precise and accurate evaluation of the spermatozoa with a high level of objectivity, allowing the improvement of the process of seminal evaluation in animals (ARRUDA, 2000; VERSTEGEN et al., 2002). However, is still not clear whose characteristic of sperm movement determined by CASA is able to predict fertility or fertility rate (FERREIRA et al., 1997; AMANN, 1989).

Recent advances in stain technology have provided new approaches to assess the functional capability of the spermatozoa in several animal species (GARNER; JOHNSON, 1995; ARRUDA et al., 2002b; ARRUDA; CELEGHINI, 2003; CELEGHINI et al., 2004; CELEGHINI, 2005). Therefore, functionality or integrity of sperm structures is monitored by fluorescent probes, which has the ability to bind to specific sites in the cell, allowing a direct and easy diagnostic, depending on their physical characteristics (CELEGHINI, 2005). The combination of various fluorescent stains makes possible the evaluation of several cell structures simultaneously. The association of the PI, FITC-PSA and Mito Tracker Green FM probes has been used to assess at the same time the integrity of plasmatic and

acrosomal membranes, and mithocondrial function in bovine semen (ARRUDA; CELEGHINI, 2003) and equine (ARRUDA et al., 2002; CELEGHINI et al., 2004). The same parameters are evaluated in the association of PI, H342, FITC-PSA and CMXRos; and PI, H342, FITC-PSA and JC-1 (CELEGHINI, 2005).

Although the use of fluorescent probes in microscopy is an effective method to sperm evaluation, the usually number of examined cells in one single analysis is no more than 200. Flow cytometry has becoming a more desirable technique than the classical methods since this automated system examines 10,000 sperm cells in one minute, resulting in more confidence of results (ARRUDA, 2000). For this reason, flow cytometry has been more and more employed in the assessment of the spermatic characteristics on several mammal species (EVENSON, 1980; GARNER; JOHNSON, 1995).

ROS production leads to the occurrence of lipidic peroxidation in the spermatozoa, causing the accumulation of hidroperoxids in the sperm membrane, forming later the malondialdehyde (MDA), that stays in corporal fluids, and that can be use used as a marker of lipidic peroxidation. Among the different analytical methods, the reaction with the 2-thiobarbituric (TBA) is the most used, where the MDA reacts with TBA producing a compound that can be measured by absorbance and fluorescence, being these products called thiobarbituric acid reactive substance (TBARS) (JANERO, 1990). The fluorescent probe C11-BODIPY$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1 ,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) is an analog of unsaturated fatty acids that is incorporated to the cellular membrane. It is observed a red fluorescence of 580-620 nm wavelength while this fluorophore is nonoxidized form. When ROS is present, there is a changing in the fluorescence from red to green, which wavelength is 495 to 545 nm (**BALL; VO, 2002**).

With regards to the seminal plasma proteins, Killian et al. (1993) detected four fertility-associated proteins in the bull, being two proteins (26 kDa e 55 kDa) prevail in high fertility bulls and two other proteins (16 kDa and 16 kDa) predominate in low fertility bulls. Later, Cancel et al. (1997) identified the 55 kDa protein predominant in semen of higher fertility bulls as being osteopontin (OPN). At the same way, Gerena et al. (1998) identified the 26 kDa protein as being the lipocalin type prostaglandin D sintetase (L-PGDS). Although Roncolletta et al. (1999) found a high incidence of a protein with approximately 61.8 kDa in high freezibilty Gir semen. Despite several studies indicate the seminal plasma proteins as markers, much work has to be conducted to answer the question about the myth and reality of the fertility markers (SULLIVAN, 2004).

Even though scientists strongly look forward to develop laboratorial techniques that can exactly predict the semen fertility, none of these tests can solely estimate the fertilization potential. This goal is of difficult achievement because the necessity of the spermatozoa to have different attribute to the egg fertilization. Is important to consider the importance of semen quality in order to obtain good fertility rates, mainly in FTAI and FTET programs (ARRUDA et al., 2004)

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NEW ADVANCES IN TREATMENTS OF BOVINE EMBRYO DONORS AND RECIPIENTS

Baruselli, P.S.[1]; Martins, C.M.[1]; de Sá Filho, M.F.[1]; Nasser, L.F.[1]; Gimenes, L.U.[1]; Madureira, E.H.[1]; Bó, G.A.[2]

[1]Departamento de Reprodução Animal, FMVZ-USP, Rua Prof. Orlando Marques de Paiva, 87, 05508-000, São Paulo-SP, Brasil. barusell@usp.br [2]Instituto de Reproducción Animal Córdoba, J.L. de Cabrera 106, X5000GVD Córdoba e Facultad de Ciencias Agropecuarias, Universidad Católica de Córdoba, Argentina

Introduction

In the last years Brazil has been presented great number of *in vivo* and *in vitro* embryo transfers, contributing with almost 50% of the total embryos produced in the world. In Brazil and other tropical countries there are an increasing demand to genetics multiplication of *Bos indicus* and *Bos Taurus* females. However, there are important differences in the physiology and in the reproductive behavior among these species, which can restrict the efficiency of multiple ovulation and embryo transfer (MOET) programs.

The objective of this paper is to present the results of the most recent researchs about superstimulation treatments, aiming to control follicular growth and ovulation, making possible fixed-time artificial insemination (FTAI) in *Bos indicus* and *Bos taurus* donors. Also, treatments to synchronization of recipients for fixed-time embryo transfer will be discussed.

Superovulation with fixed-time artificial insemination in *Bos indicus*

Several studies evaluated the ovulatory response in cows superstimulated with treatments that control the time of ovulation (RIEGER et al., 1990; D'OCCHIO et al., 1997, CARVALHO et al., 2000; NOGUEIRA et al., 2002; ZANENGA et al., 2003; BARUSELLI et al., 2003 and MARTINS et al., 2005).

In Nelore cows, NOGUEIRA et al. (2002) compared the efficiency of superstimulatory protocols using deslorelin auricular implant or progesterone-releasing intravaginal device (CIDR). Ovulations were induced by LH administration, 48 or 60 hours after $PGF_{2\alpha}$ treatment (in deslorelin treatment, LH was only injected 60 hours after PGF2α). FTAI was performed 10 and 20 hours after LH injection. The number of viable embryos and pregnancy rates after embryo transfer were similar between the groups.

Considering negative effects of high plasma progesterone concentrations near to ovulation in superstimaled cows (DIELEMAN and BEVERS, 1993), our research group designed an experiment to evaluate the anticipation in progesterone-releasing intravaginal device withdrawal, in superstimulated Nelore cows submitted to fixed-time artificial insemination (SOFT). For this purpose, ten Nelore cows (n=10) kept on pasture in Campo Grande-MS were randomly assigned in two treatments ("cross-over"

design), being treatments changed 35 days later. In the first group (P24), progesterone-releasing intravaginal device removal (administered at the same time to the sixth FSH dose) and administration of 25mg of LHp (Lutropin, Vetrepharm, Canada) occurred 24 and 48 hours after PGF2α injection, respectively. The second group (P36) received similar protocol to the P24 Group, except by progesterone-releasing intravaginal device withdrawal time, that was performed 36 hours after PGF2α administration. All donors were inseminated 12 and 24 hours after LH. The embryo recovery and classification was performed seven days after LH (ZANENGA et al., 2003).

36 superstimulation protocols in Nelore (*Bos*

4	P36
)	10
1	63.4
15.1	17.7 ± 11.7
7.5	10.3 ± 5.9
4.8	5.2 ± 6.1
8.7	2.2 ± 3.5

Results (Table 1) are suggestive that progesterone-releasing intravaginal device withdrawal 24 or 36 after PGF2α, followed by LH ovulation induction (48h after PGF2α injection) and FTAI 12 and 24 hours after LH is a viable protocol for MOET programs in *Bos indicus*, without estrous detection and making possible the programming of synchronization activities, embryo recovery and transfer.

Aiming to evaluate the superstimulatory response in Nelore cows to reduced dosages of FSH (100 versus 133 versus 200mg; Folltropin-V, Vetrepharm, Canada), our group (Baruselli et al., 2003) used the experimental "cross-over" design in order to prevent individual effects in the treatments response. Results indicate that there are no differences in the superovulatory responses relative to number of transferable and suitable for freezing embryos after using 100, 133 or 200mg of FSH in supestimulated Nelores donors.

Recently, our research group has beginning studies using eCG in SOFT protocols in *Bos indicus* (Chiari et al., 2005). Results are suggestive that although the number of viable embryos has undergone numeric reduction (4.4 ± 1.3 vs. 2.9 ± 1.0; $p>0.05$), the eCG treatment reduces the animal handling (8 FSH vs. 1 eCG administrations) in SOTF protocols.

Superstimulation with fixed-time artificial insemination in high production Holsteins cows

The SOFT protocols developed until the moment present good results in *Bos indicus* cows, however, when *Bos taurus* cows are submitted to such procedures, the number of recovered structures has been unsatisfactory. There are several physiological peculiarities that differentiates *Bos indicus* and *Bos taurus* cows, such as the follicular diameter deviation, that deserves attention. It was verified that *Bos taurus* females reaches largest diameter on deviation than *Bos indicus* females (6.2mm; GIMENES

et al., 2005 for *Bos indicus* and 8.5mm; GINTHER et al., 1996 for *Bos taurus*). Thus, it would be supposed that *Bos taurus* follicles need largest follicular diameter to acquire ovulatory capacity. Sartori et al. (2001) verified that *Bos taurus* females ovulated after LH administration only when follicular diameter was greater than 10mm. Recently, GIMENES et al. (2005) had verified that *Bos indicus* females already reaches high ovulatory capacity when diameter was ≥ 8.5 mm. In this study the percentage of heifers that ovulated after LH treatment was: 33.3%[a] (3/9); 80.0%[b] (8/10) and 90.0%[b] (9/10; $P<0.05$) for groups 7 to 8.4 mm; 8.5 to 10 mm and > 10mm, respectively. Therefore, protocols that delay the administration of ovulation inducer, allow follicular growth until diameters correspondent to ovulatory capacity, improving the efficiency of the superstimulatory programs in *Bos taurus*.

In order to test this hypothesis, our research group (Rodrigues et al., 2005), had used different SOFT protocols in 40 high production Holstein cows (mean of 39.0 L). The aim was to verify the effect of 12 hours delay in the ovulation induction (thus, increasing ovulatory rates) and also the effect of insertion of one or two Norgestomet auricular implants during the superstimulatory treatment (in order to reduce the LH pulsatility). The addition of two Norgestomet auricular implant did not influence the analyzed variable, but the 12 hours delay in the administration of ovulation inducer reduced the number of follicles present at the time of superstimulatory treatment ($P<0.05$). In despite of this, the number of structures recovered, as well as the number of transferable embryos and embryos suitable for freezing did not increase statistically. Although a statistically significant increase was not verified, the use of two Norgestomet auricular implants (1.5 ± 0.5 versus 3.0 ± 1.0) and the 12 hours delay in ovulation induction (1.6 ± 0.6 versus 2.9 ± 1.0) had improved the number of embryos suitable for freezing. Thus, it is possible that these strategies increase the superstimulation protocols efficiency in high production Holstein cows.

In sequence, Martins et al., (2005) evaluated the effect of the progesterone-releasing intravaginal device permanence (24 or 36 hours after PGF administration) and the time of the ovulation inducer administration (48 or 60 hours after PGF) in the follicular dynamics and embryo production in high production Holstein cows submitted to SOFT. The hypothesis of this experiment was that a delay at the time of progesterone-releasing intravaginal device withdrawal and the exogenous ovulation induction would promote better synchronization of ovulation and, consequently, greater amount and quality of viable embryos. A total of twelve (n=12) Holstein cows were kept under the same management and feeding conditions. The animals were assigned in four groups, in a Latin square design (2 X 2factorial). All cows were inseminated with the same ejaculate of only one sire. No time of P4 permanence effect was observed in the analyzed variables. There was effect of time of LH administration in the number of >8mm follicles at LH ovulation induction, number of transferable embryos and embryos suitable for freezing, and first to last ovulation interval. Possibly, the 12 hours delay in the LH administration promotes a higher follicular growth, reduces the ovulations widespread and increases the number of transferable and suitable for freezing embryos. Results are indicative that LH treatment 60 hours after PGF administration increases the SOFT efficiency of high production Holstein cows.

In recent study, Martins et al., (2005, preliminary data) also evaluated the effect of the 12 hours delay on ovulation induction in superstimulated Nelore cows. Conversely to observed in *Bos taurus*, the 12 hours delay on ovulation induction did not present satisfactory results in *Bos indicus*, increasing

(P=0.06) the number of degenerated embryos and promoting numerical reduction of transferable and suitable for freezing embryos. These preliminary data indicate that, probably, the LH administration 48 hours after PGF2α is appropriate to induce ovulation in Nelore (*Bos indicus*) cows submitted to SOFT.

Recipients synchronization for fixed-time embryo transfer

In general, the commercial programs of embryo transfer present low proportion of recipients selected/treated. Usually, in a group of recipients treated with PGF associated to estrous detection, only 40 to 50% of treated animals are used to embryo transfer. Considering conception rates of 50% in all animals treated, only 20 to 25% of pregnancies are obtained in the end of the program. Therefore, the increment of the selected/treated proportion and conception rates in embryo recipients is fundamental to maximize pregnancy rates, increasing the genetic and economic return of embryo transfer program, determining greater number of calves/year and also reducing costs with no pregnant recipients.

Studies demonstrate the relation among plasma progesterone concentrations and conception rates (Binelli et al., 2001; Thatcher et al., 2001; Baruselli et al., 2003; Reis et al., 2004). The increase in plasma progesterone concentrations during diestrus was positively correlated with embryonic development, for the greater amount of nutrients available to uterine lumen (Geisert et al., 1992), and also for increasing the interferon-τ secretion by conceptus. (Kerbler et al., 1997; Mann & Lamming, 2001), which promotes reduction of the PGF2α secretion by uterine endometrium and blocks the luteolisis (Wathes et al., 1998). This phenomenon was associated to increase in conception rates (Fuentes & De La Fuentes, 1997, Baruselli et al., 2000).

Inquiries performed by our group aiming superstimulate embryo recipients for fixed-time embryo transfer (Baruselli et al., 2000c and 2001, Nasser et al., 2004, Reis et al., 2004), demonstrated positive correlations among number of corpora lutea, plasma progesterone concentration and conception rates after embryo transfer. These works indicated that eCG treatment at expected time of emergence of the new follicular wave was efficient to induce superovulation or to increase the dominant follicle diameter. These effects determine greater number of CL (or greater diameter of the single CL), higher plasma progesterone concentration, greater selected/treated proportion, conception and pregnancy rates than traditional treatments with prostaglandin and estrous observation. Moreover, they make possible the fixed-time embryo transfer, without estrous detection, and with previous programming of the best day for the embryo transfer.

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ROUND TABLE

Problems related to commercial IVP embryos

COMMERCIAL *IN VITRO* FERTILIZATION CHALLENGES

Queiroz, L.M.V.; Peixer, M.A.S.; Malard, P.F.; Santana, G.M.; Xavier, M.C.; Sousa, R.R.

BIO – Biotecnologia em Reprodução Animal Ltda., SMPW Quadra 5 Conj. 05 Lote 01 Casa C - Brasília-DF, luismauro@biotecnologiaanimal.com.br

Introduction

The *in vitro* embryo production in a commercial large scale program presents certain operational particularities, which make this commercial activity, a different model from *in vitro* production system, which are under academic laboratories located at state institutions. The success of a commercial *in vitro* fertilization program has passed through several barriers, such as: commercial relationship with clients, recipient selection, and transfer procedure of produced embryos. In addition to challenges encountered during the lab routine. Those points make the IVF, a technique linked to many traps, which without attention and good sense, might lead to low results. This article aims to present the critical aspects on bovine *in vitro* embryo production in a commercial program based on the BIO Lab's point of view.

Bovine *in vitro* embryo production – Election of an efficient protocol for IVF

Different IVF protocols are reported in the literature, and each of them presents particularities and respective results, but with the same dorsal spine*: in vitro* oocyte maturation, sperm preparation, fertilization and *in vitro* culture. The differences encountered among those protocols, from a diversity of commercial labs are the attempts in achieving all of those challenges.

The Bio lab, located in Brasilia – DF, has a large client market, which is not limited to the Federal District area or only to the state of Goiás. Thus, an efficient occyte transport system has been created, which allows 16 hours from the start of follicular aspiration to the arrival at the lab. The influence of time over *in vitro* embryo production has been investigated, and the data related to intervals from aspiration to lab arrival were recorded for further analysis: Group 1 (1 to 2 hours), Group 2 (3 to 5 hours), Group 3 (6 to 9 hours), and Group 4 (10 to 16 hours). The embryo production rates were: G1 (48%), G2 (38%), G3 (40%), and G4 (36%).

Another challenge was then presented: the establishment of an embryo transport protocol that would not significantly interfere with the pregnancy rates. A stored system for embryo transport was developed, allowing 18 hours from stored embryo to transfer. No statistical differences were observed when G1, G2 and G3 were considered, and the pregnancy rates were respectively: 41%, 40% and 37% for these groups. However, there was a significant difference when comparing those rates with a 34% rate in G4.

When considering the oocyte retrieval rates in relationship to pregnancy rates, it was possible to observe a statistical difference when comparing G1(20%) and G 2, 3 and 4 (16%, 15% and 12%)

respectively. Even though, a difference regarding embryo production and pregnancy rates were observed, G4 presented a positive commercial index, which means that the pregnancy costs were at acceptable levels even for this group.

Functional Semen Particularities

The *in vitro* fertilization process starts with sperm separation, which is a necessary approach to remove dead sperm, seminal plasma, and cryoprotectants. The thawed sample, normally is submitted to centrifugation, and the live sperms are exposed to a density gradient, and all the undesirable material removed. However, the centrifuge process can promote damage to the sperm plasma membrane and acrossome, consequently altering its chromatin content.

Some bulls, which are commercially available, and present an excellent pregnancy rates when used for artificial insemination are known to suffer those deleterious effects when used for IVF, resulting on a low fertilization, cleavage and embryo production rates.

The IVF environment can have direct effect on sperm performance, requiring special care. Distinct effects can be found on bulls, and some of those are: IVF media, Heparin concentration, and the use of antioxidants, with enormous difficult in establishing a single protocol for all routine lab.

In vitro culture

Several models of *in vitro* culture are available, with excellent results being published in the literature. However, when considering a commercial model, some alternatives, such as, the use of an intermediate host, or a sophisticated use of a low O2 atmosphere are not practical for this purpose. The *in vitro* culture protocol must be efficient and simple.

Transfer of *in vitro* produced embryos

One factor that interfere the most with a final result at an IVF program, is the recipient quality during the embryo transfer procedure. Some points must be considered when ideal conditions for recipients are discussed: 1) efficient sanitary control to avoid an entrance of infectious diseases at the lab, as brucellosis, tubercullosis, leptospirosis, IBR, BVD, and clostridial infection. 2) ideal strategies regarding nutrition, such as the use of mineral supplementation, specially during the dry season. 3) different protocols for recipient estrus synchronization are established, and they present distinct objectives. Depending upon recipient availability and frequency of IVF sessions, the following methods can be used: a single prostaglandin administration, which presents an estrus synchronization rate of 40%, but with a low cost; b) progestagen implant + estrogen + prostaglandin, with an estrus synchronization rate of 65%, on treated animals. c) progestagen implant + estrogen + prostaglandin + eCG, which presents the best estrus synchronization rates, however with the highest cost. This protocol is recommended for use in the recipients in a sequential program or at farms where the recipients are restricted.

Characterization of donor cows submitted to follicular aspiration

The donors that are candidates to become part of an IVF program, normally have pre-requirements, such as: recognized genetic merit, proved reproductive performance, good performance during breed judgment, high financial value, expected sale in a short period of time, and acquired reproductive pathology. Except for the last point cited, this picture puts the donor cow in a special position for treatment, normally followed by nutritional management characterized by a high protein, energy, and stall maintenance; which result in a weight gain, with excess fat deposition. This condition is not desirable for an IVF candidate, leading to the decreased on viable oocyte numbers (increase on denude and degenerated oocytes) and decrease on *in vitro* produced embryo rates. However, donors with acquired reproductive pathologies, except during anestrus, present irregular oocyte production rates, embryos and pregnancy rates that would justify the use of this technique.

Conclusion

Several applications for *in vitro* fertilization can be pointed, as the increase of productivity index and a strong national livestock market, which contributes day by day for the technical expansion of programs involving animal husbandry at several regions in Brazil.

Saliba W.P. & Alvim, M.T.T. 2005. **Problems related to comercial *in vitro* embryo production**. *Acta Scientiae Veterinariae*. 33 (Suppl 1): 162-166.

PROBLEMS RELATED TO COMERCIAL IN VITRO EMBRYO PRODUCTION

Saliba, W.P.; Alvim, M.T.T.

Cenatte Embriões Ltda, Rua Dr. Rocha, 832 – Caixa Postal 64 - Pedro Leopoldo – MG
wilson@cenatte.com.br

The majority of problems in a commercial *in vitro*-produced embryo system is associated with culture process. Numerous factors are known to influence their intensity of occurrence, such as the use of non-defined media for *in vitro* produced (IVP) embryos. On a daily basis, some commercially important problems can be present in a IVP practice. Some of those are the occurrence of large calf syndrome, high abortion rates, natal death, large number of male fetuses, IVP embryos less suitable for freezing, and the limited use of sexed semen. The first week post-partum was closely monitored in 215 recipients cows from different breeds attempting to analyze and figure the problems encountered during gestation and birth from pregnancies of IVP embryos. The follow up from our clients made possible to verify the percentage of abortion, natal-death, normal parturition, dystocias, hydrops, and large calf syndrome. From the 215 pregnancies, 80% of the recipients had a normal parturition (172/215), 3.7% (8/215) aborted between 60 and 120 days of gestation, and 7.4% (16/215) aborted between 120 and 260 days, with a total abortion rate of 11.2% (24/215). The natal-death was 7.0% (16/215) with 3.3% (7/215) of dystocias. In addition, large calf syndrome associated with hydrops was observed in one case (0.5% - 1/215). The percentage of males and females obtained from 2699 IVP pregnancies were verified from May 2003 to March 2005. Eighty three pregnancies were obtained from hatched blastocysts (HB), 2128 from expanded blastocysts (XB), 296 from blastocysts (BL), 90 from initial blastocysts (IB) and 102 from morula (MO). The percentage of males obtained were respectivally: 36.1% (HB), 53.7% (BX), 59.1% (BL), 53.3% (BI) and 57.8% (MO). When all embryonic phases were considered, 53.9% of males and 46.1% of females were observed. In the same period, the frequency of male and female pregnancies were evaluated for embryos produced by embryo-transfer following superovulation (ET-SOV). From 3859 pregnancies, 51.6% were males and 48,4% were females. More male pregnancies were observed in IVP when compared to ET- SOV. In our system of *in vitro* produced bovine embryos, the dada suggest that advanced embryonic structures as BH and BX have a low tendency to result in males when compared to BL and MO. Regarding different breeds, the number of pregnancies and respective breeds were: 412 from Brahman, 124 from Gir, 542 from Guzera and 1507 from Nelore. And the percentages of male pregnancies for each breed mentioned above were respectively: 54.4%, 54.8%, 52.4% and 53.9, which demonstrate that the tendency of male pregnancies seems not to be breed related.

Another problem related to *in vitro* embryo production is the results variability obtained with embryo cryopreservation, principally when slow freezing method is considered. In addition, the lack of repeatable results does not allow its commercial use. To evaluate our protocol, embryos were cryopreserved using slow freezing. Those embryos were classified as being grade I expanded blastocysts

at day 7 of culture. Ethylene glycol (1.5 M) was used as a cryoprotectant, and after being stored for a determined period, embryos were thawed and exposed to SOFaaci in culture drops at a controlled environment, the rates of expanded and hatched structures were evaluated at 24 and 48 hours in culture. From 51 thawed embryos, 27.5% were re-expanded within 24 hours and only 9.8% hatched within 48 hours. These results demonstrate a need for further research in this area. Embryo quality improvement can favor IVP embryos in their capacity to better resist cryopreservation leading to commercially compatible pregnancy results. Later on, 20 expanded blastocysts were frozen at the same conditions mentioned above. Eighteen embryos were thawed and transferred to synchronized recipients resulting in 3 pregnancies (16.7% of pregnancy rate), which were confirmed at 45 days of gestation by ultrasound. The sexed semen availability in the market allowed Cenatte to participate in trials, which had the objective of verify sexed semen performance at a commercial IVP program. Sexed semen from two Nelore bulls (A and B) and non-sexed semen from a third control Nelore bull (C),were used. Grade I,II, and III oocytes were obtained by ovum pick-up (OPU) and submitted to maturation in modified TCM 199 at CO_2 5%, 39°C with saturated humidity. The *in vitro* fertilization (IVF) was performed in 190, 73 and 179 oocytes using semen from respective bulls: A,B and C. Semen ($2x10^6$ sptz/ml) was submitted to Fert-TalP medium supplemented with BSA, Piruvate, PHE and Heparin. After thawing semen was submitted to a Pecoll gradient. The IVF was performed at the same atmosphere mentioned above for IVM and the *in vitro* culture (ICV) was performed using SOFaaci medium supplemented with a protein source at 5% CO_2, 5% O_2, 39°C and saturated humidity during 6 days. Bulls, cleavage rates and produced embryos were respectively: A (64,7% e 37,4%), B (61,6 e 26%) and C (52% e 30,7%). A diference among bulls was observed regarding embryo production. Those results suggest that sexed semen can be used in IVP based on embryo yielding , which are considered commercialy compatible. Following the same IVP conditions previously described in this paper, commercial embryo production using sexed semen was carried out. Five bulls from different breeds, Holtein (HO), Nelore Mocho (NO), Brahman (BR), Nelore (NE) and Girolando (GO) were used. Oocytes (n = 226) were obtained from 10 donnor cows by OPU and submitted to IVF using semen from the cited bulls. Clivage rates and embryos were evaluated respectively at day 2 and day 7 of IVC, considering D0 as the day of IVF. At 60 days from IVF the pregnancy check was performed followed by fetal sexing. Results from IVF using the GO bull semen are not presented due to contamination after the procedure. The cleavage rates were 100% (14/14), 100% (66/66), 77% (46/60) and 50% (43/86), respectivelly for HO, NO, BR and NE bulls. The rates of tranferable embryos were: 57, 46, 33 and 53%, respectivelly for HO, NO, BR and NE bulls. The average for embryo production was 45% (102/222), and the pregnancy rate was 45% (46/102), with 83% of females being diagnosed by fetal sexing at pregnancy check.

Bulls	Oocytes	Cleavage		ET		Pregnancy		Female	
Lacrose	14	14	100%	7	50%	4	57%	3	75%
Bacana	66	66	100%	52	79%	24	46%	20	83%
Pillar	60	46	77%	24	40%	8	33%	6	75%
Edhank	86	43	50%	19	22%	10	53%	9	90%
Total	**226**	**169**	**75%**	**102**	**45%**	**46**	**45%**	**38**	**83%**

Bull	Viable	Cleavage		IVF embryos		ET embryos		Pregnancy		Male		Female	
	Oocytes	N	%	N	%	N	%	N	%	N	%	N	%
Lacrose	14	14	100.0	7	50.0	7	50.0	4	57.1	1	25.0	3	75.0
Bacana	66	65	98.5	64	97.0	52	78.8	24	46.2	4	16.7	20	83.3
Pillar 30	60	46	76.7	28	46.7	24	40.0	8	33.3	2	25.0	6	75.0
Edhank	86	22	25.6	23	26.7	19	22.1	10	52.6	1	10.0	9	90.0
Total	226	147	65.04	122	54	102	45.13	46	45.1	8	17.39	38	82.61

G	Trials	Total IVM	Total Emb.	% Emb.	% Emb.GI	Cliv.	% Cliv.
1	Galante - 4 trials / S	190	71	37,4	23,7	123	64,7
2	Aldebaran - 2 trials / S	73	19	26,0	13,7	45	61,6
3	Joquei - 3 trials - not S	179	55	30,7	20,7	93	52,0

Pregnancy rates by breed – 2003 to 2005

Breed	Total TE	Pregnancy	%	Female	Male	Total	%Male
BLONDE	6	5	83.3	1	4	5	80.0
BRAHMAN	**1084**	**418**	**38.6**	**188**	**224**	**412**	**54.4**
CACHIM	36	20	55.6	11	9	20	45.0
GIR	**346**	**133**	**38.4**	**56**	**68**	**124**	**54.8**
GIROLANDO	21	13	61.9	6	7	13	53.8
GUZERÁ	**1286**	**552**	**42.9**	**258**	**284**	**542**	**52.4**
HOLANDESA	6	2	33.3	0	2	2	100.0
NELORE	**3562**	**1555**	**43.7**	**695**	**812**	**1507**	**53.9**
NELORE MOCHO	151	77	51.0	29	45	74	60.8
TOTAL	**6498**	**2775**	**42.7**	**1244**	**1455**	**2699**	**53.9**

BREED	Total TE	Pregnancy	%	Female	Male	Total	% Male
BEI	220	84	38.2	53	30	83	36.1
BXI	4228	1953	46.2	889	1017	1906	53.4
BHI	392	190	48.5	72	115	187	61.5
BXI and BHI	4620	2143	46.4	961	1132	2093	54.1
BXII	128	37	28.9	24	10	34	29.4
BXIII	9	1	11.1	0	1	1	100.0
TOTAL BX and BH	4748	2180	45.9	985	1143	2128	53.7
BLI	806	290	36.0	111	165	276	59.8
BLII	96	21	21.9	10	9	19	47.4
BLIII	9	1	11.1	0	1	1	100.0
TOTAL BL	911	312	34.2	121	175	296	59.1
BiI	215	73	34.0	29	40	69	58.0
BiII	66	16	24.2	8	7	15	46.7
BiIII	23	6	26.1	5	1	6	16.7
TOTAL Bi	304	95	31.3	42	48	90	53.3
MOI	222	81	36.5	30	49	79	62.0
MOII	65	19	29.2	11	7	18	38.9
MOIII	26	5	19.2	2	3	5	60.0
TOTAL MO	313	105	33.5	43	59	102	57.8
TOTAL	6496	2776	42.7	1244	1455	2699	53.9

IVF Frozen Embryos - *In vitro* Trial - 18/04/2005

Embryos		Evaluation - 24 hs				Evaluation- 48 hs			
Phase	Nº	Reexpan.	Eclodin./ Eclodido	% Rees.	% Eclo.	Reexpan.	Eclodin./Eclodido	% Rees.	% Eclo.
BX I	8	5	0	62.5	0.0	0	2	0.0	25.0
BX I	10	0	0	0.0	0.0	0	0	0.0	0.0
BX I	11	0	0	0.0	0.0	0	0	0.0	0.0
BX I	17	6	0	35.3	0.0	0	1	0.0	5.9
BXI NI	5	3	0	60.0	0.0	0	2	0.0	40.0
Total BX I	51	14	0	27.5	0.0	0	5	0.0	9.8
Total MO I	3	1	0	333	0.0	1	0	33.3	0.0
Total BL I	2	0	0	0.0	0.0	0	0	0.0	0.0
Total Bi I	1	0	0	0.0	0.0	0	0	0.0	0.0

Location	Female	Male	Total	% Male
CALCIOLANDIA 2003	341	363	704	51.6
SANTA FÉ 2003	702	739	1441	51.3
SANTA FÉ 2004	386	440	826	53.3
JAGUARA 2004	437	451	888	50.8
TOTAL	1866	1993	3859	51.6

Students Competition

EFFECT OF NITRIC OXIDE ADDICTION IN BOVINE OOCYTE DURING MATURATION IN VITRO

Viana, K.S.; Caldas-Bussiere, M.C.; Matta, S.G.C.; Paes de Carvalho, C.S.; Faes, M.R.; Quirino, C.R.

Setor de Reprodução Animal, LMGA, CCTA, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, 28013-602, Brasil. kelensv@uenf.br

Nitric oxide (NO) is highly reactive free radical, that is involved in intra and intercellular signaling in some stages of reproduction. The objective of the present study was to evaluate the effect of the addition of sodium nitroprusside (SNP), NO donor, on nuclear and cytoplasmic in vitro maturation of bovine oocytes. Bovine ovaries were collected at local abattoir. Oocytes were aspirated from 3-5mm follicles. Only oocytes with homogeneous cytoplasm and at least three layers of cumulus cells were used. SNP was used at 10^{-9}, 10^{-7}, 10^{-5} e 10^{-3} M. To determine stage of meiosis, oocytes were staingin in 2% acetic orcein. Cortical granules were stained with conjugated Lens culinares-FITC. Analysis of variance was carried out and the means were compared by *t* test at a level of 5%. Low concentrations of SNP (10^{-9}, 10^{-7} and 10^{-5} M) had no significant effect on nuclear maturation, however, when a high concentration of SNP (10^{-3} M) was added/retarded nuclear maturation ($p<0.05$), with oocytes remaining in metáfase I (MI, 97,5±3,1%) after 24 h culture. To evaluate if this effect was reversible and if a retardation or inhibition had occurred of the progression from MI to MII, oocyte were cultured in presence of 10^{-3} M of SNP for 48 h with change of way (without SNP) or not. After 48h, the oocyte had remained in MI (MI, 100%) even when the medium was out changed at 24 h without SNP or not. After 48h, the oocyte had remained in MI even when the medium was out changed at 24 h without SNP or not. The kinetics of the nuclear maturation was carried to evaluate if there had been or not a retardation in the progression of meiosis with the concentration of 10^{-3} M SNP. This concentration delayed the germinal vesicle breakdown at 8 h of culture (VG, 80,3±12,8%-$p<0.05$), and at 12 h there was no significant difference between the control and the treated group (VG, 6,0±6,1% e 19,2±8,5% respectively). The concentrations that did not induced alteration in the nuclear maturation were evaluated for cytoplasmic maturation (10^{-9}, 10^{-7} and 10^{-5} M). The concentration of 10^{-5} M improved the percentage of total migration cortical granules (94,1±6,8%-$p<0,05$), and had significant effect in the percentage of blastocysts (40,4±15,9%) superior to the control group (78,1±11,4% e 27,2±6,0% respectively). These results a indicate that the nitric oxide can have both stimulatory and inhibitory effect on the maturation of bovine oocyte in vitro, depending on its concentration.

INFLUENCES OF INDOLE-3-ACETIC ACID ON THE DEVELOPMENT OF CAPRINE PREANTRAL FOLLICLES

Matos, M.H.T.[1]; Martins, F.S.[1]; Santos, R.R.[1]; Luque, M.C.A.[2]; Silva, J.R.V.[1]; Celestino, J.J.H.[1]; Báo, S.N.[2]; Figueiredo, J.R.[1]

[1]Laboratory of Manipulation of Oocytes Enclosed in Preantral Follicles, LAMOFOPA, Faculty of Veterinary, State University of Ceara, Av. Paranjana, 1700, Fortaleza - CE, 60740-000, Brazil. htmatos@yahoo.com; [2]Laboratory of Electron Microscopy, Department of Cell Biology, University of Brasilia, Brasilia, DF, Brazil

The role of growth factors and hormones in the regulation of preantral follicle development remain poorly understood. The aims of the present study were to evaluate the effects of Indole-3-acetic acid (IAA) on survival, activation and growth of caprine preantral follicles using histologic and ultrastructural studies. To this end, pieces of caprine ovarian cortex were cultured for 1 or 5 days, at 39°C in an atmosphere containing 5% CO_2, in Minimum Essential Medium (MEM) (Cultilab, Rio de Janeiro, Brazil) supplemented with insulin, transferrin, selenium, pyruvate, glutamine, hypoxanthine, BSA, penicillin, streptomycin and fungizone and with or without different concentrations of IAA (10, 20, 40 or 100 ng/mL). Culture medium was replaced by fresh medium each two days. A small fragment from each non-cultured ovarian tissue as well as from those cultured for 1 or 5 days in a specific medium was removed for transmission electron microscopy (TEM) and the remainder was processed for classical histology to evaluate the morphology of the caprine preantral follicles. Follicles were classified as primordial or developing (intermediate, primary and secondary), as well as in normal or degenerated, depending on the morphology of the oocyte and the granulosa cells. The percentage of morphologically normal follicles and the percentage of primordial and developing follicles at each period of culture were analyzed by Chi-square test and comparisons of oocyte and follicle diameters among control and cultured groups were analyzed by Mann-Witney ($P < 0.05$). The results showed that MEM alone or supplemented with IAA (20 ng/mL) increased significantly the percentage of morphologically normal follicles when compared with other treatments. It was found that IAA at 20 or 40 ng/mL affected positively the proportion of primordial follicles that entered the growth phase after 5 days of culture. In the presence of 10 and 20 ng/mL of IAA, there was an increase in diameter of both oocyte and follicle on day 5 of culture. Although the good results observed with classical histology, the TEM showed that the concentration of 20 ng/mL of IAA did not maintain the morphological integrity of caprine preantral follicles after 5 days of culture. In conclusion, this study demonstrated that IAA at 20 ng/mL can stimulate activation and growth of caprine preantral follicles after 5 days culture. However, in the same conditions, culture of preantral follicles enclosed in fragments of caprine ovarian cortex was not effective in maintaining the ultrastructural integrity of these follicles.

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MATURATION AND VITRIFICATION OF EQUINE OOCYTES IN MEDIUN WITH EQUINE GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I.

Curcio, B.R.[1]; Deschamps, J.C.[1]; Nogueira, C.E.W.[2]; Pereira, G.R.[3]; Macedo Jr, M.C.[1]; Boff, A.L.N[1]; Rambo, G.[1]; Lucia Jr, T.[1]

[1]Biotechnology Center-UFPel; [2]Department of Veterinary Clinical – FV - UFPel, Campus Universitário s/n 96010-900,Pelotas-RS. [3]Departament of Population Health & Reproduction, School of Veterinary Medicine, UCDavis, California 95616. enderlebr@terra.com.br

The objective of this study was to investigate the effects of equine growth hormone (eGH) and its relationship with insulin-like growth factor-1 (IGF-1) on the *in vitro* maturation (IVM) and development of equine oocytes. Additionally, we also evaluated the morphological and structural integrity of all equine oocytes subjected to IVM and cryopreservation with synthetic copolymer X-1000 (SuperColl X-1000™; 21st Century Medicine). Complex *cumulus oophorus* (COCs) were cultured in TCM199 supplemented with 0.1% BSA and antibiotics. COC's (n=122) were placed in a four-well dish supplemented according to four treatments, as follows: a) control, no additives (n=34); b) 400 ng/mL of eGH (n=31); c) 200 ng/mL of IGF-1 (n=35); and d) 400 ng/mL of eGH + 200 ng/mL of IGF-1 (n=22). After maturation, oocytes were fixed (n=37) or subjected to the vitrification protocol (n=85). Cryopreserved oocytes were exposed to 1,4 M dimethyl sulfoxide (DMSO) + 1,8 M ethylene glycol (EG) + 1% X-1000 for 3 min, and then transfer to 2,8 M DMSO + 3,6 M EG + 0,6 M sucrose + 1% X-1000 for 1min. Next, the oocytes were loaded to the open pulled straw and transferred to liquid nitrogen vapor, then held in vapor for 3 sec before being plunged into liquid nitrogen. After cryopreservation, the oocytes were thawed and transferred into a PBS containing a decreasing sucrose concentration, for 5 min of each concentration: 0.5M, 0.25M, 0.125M and PBS with 0.4% BSA. Nuclear and cytoplasmic maturation status were assessed by laser scanning confocal microscopy. Differences in oocyte maturation after different treatment IVM were analyzed by Fisher's exact test. Maturation rates of oocytes were 26.1% control, 23.5% eGH, 31.8% IGF-I and 17.6% eGH + IGF-I, weren't significant difference among the treatment groups ($p>0,05$). However, the results suggest that the eGH + IGF-1 group can develop the assessment resumption of meiosis (MI+MII=86,7%). After vitrification/thawing, there weren't found oocytes with sign of lysis, membrane damage, precocious cortical granule exocytosis, swelling, vacuolization, degeneration or fragmented cytoplasm. In conclusion, the addition of eGH and/or IGF-I to our IVM protocol didn't increase rates of nuclear and cytoplasmic maturation. Oocytes subject to vitrification with X-1000 had an intact plasma membrane and didn't show any sign of vacuolization or degeneration. This is the first report showing the vitrification with X-1000 in equine oocytes.

DOES THE CYTOPLAST TRANSFER ALTERS THE EMBRYONIC DEVELOPMENT AND GESTATIONAL TERM DEVELOPMENT IN BOVINE?

Ferreira, C.R.[1]; Meirelles, F.V.[2]; Perecin, F.[1]; Méo, S.C.[3]; Tetzner, T.A.D.[1]; Saraiva, N.Z.[1]; Landim Jr., L.P.[1]; Yamazaki, W.[1]; Vantini, R.[1]; Garcia, J.M.[1]

[1]DMVPRA-FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil, [2]ZAB-FZEA-USP, Pirassununga-SP, Brazil, [3]CPDGRA-IZ, APTA/SAA, Nova Odessa-SP, 13460-000, Brazil.
chrisferreira13@yahoo.com.br

Cytoplasmic transfer (CT) has been applied in rodents in order to study nuclear-cytoplasmic interactions and mtDNA heteroplasmy inheritance (Renard *et al.*, Development, v.120, p.797-802, 1994; Meirelles *et al.*, Genetics, v.148, p.877-883, 1998), and has also been used to obtain pregnancy in a group of women with diagnosis of "recurrent implantation failure", generating discussion on the safety of this procedure and demand to evaluate its consequences in animal models. Our aim was to apply and evaluate the CT technique in bovines, a domestic species in which cytoplasmic impact in animal production and mtDNA genes selection are of interest. Oocytes obtained from slaughtered cows were *in vitro* matured (IVM). At 22h IVM, oocytes to be used as cytoplast donors were enucleated in MII and parthenogenetically activated (ionomycin 5 µM for 5 min and 6-DMAP 2 mM for 4 hours). Receptor zygotes production: Oocytes were *in vitro* fertilized (IVF) at 24h IVM. After 10-12h IVF receptor zygotes had *cumulus* cells removed and were selected for the presence of the second polar body (2nd PB). Both receptor zygotes and donor cytoplasts were placed during 30 min in medium containing 7.5µg/mL cytochalasin B before microsurgery. Receptor zygotes had their 2nd PB and surrounding cytoplasm removed prior to CT. Around 10-15% of donor cytoplasm was introduced into the periviteline space of the receptor zygote and electrofused (2 pulses of 1.5kV/cm for 30µs in 0.28M Mannitol solution). Culture medium was SOF supplemented with 2.5% FCS and 0.5% BSA. To evaluate initial segregation of cytoplast donor mitochondria (stained with MitoTracker CMX ROS®) and nuclear division (Hoechst 33342), 9 CT embryos presenting regular cleavage at 32h post IVF had the ZP removed and were analyzed under epifluorescence microscopy (330-385nm and 545-580nm). Eight of the 9 cleaved embryos had transferred mitochondria unevenly distributed between blastomeres. All blastomeres were confirmed nucleated. To evaluate CT impact on embryo development, CT embryos and IVF control group were assessed for cleavage and blastocyst rate. Data was statistically analyzed through ANOVA. Cleavage rate of CT (n=196) and IVF embryos (n=195) didn't differ (73.87 and 77.53%, respectively), but blastocyst rate was lower ($P<0.05$) in CT embryos, compared to IVF (30.06 and 38.53%, respectively). Cytoplasmic compatibility previously observed among *B. taurus* and *B. indicus* (Meirelles, Genetics, v.158, p.351-6, 2001) allowed CT involving Brazilian Nellore (mtDNA *B. taurus*) as receptor zygotes and Pure of Origin Imported (POI) Nellore (mtDNA *B. indicus*) as cytoplast donors. Eleven CT *taurus/indicus* embryos were transferred to 8 recipient cows, obtaining 2 single gestations. One male fetus gestation was interrupted at 85 days. A female calf was delivered without intervention. These results show that CT can be performed in bovine allowing development to term, and although it affects negatively embryo development, the effect is acceptable with an *in vitro* production system.

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EFFECTS OF FOLLICLE-STIMULATING HORMONE ADMINISTRATION IN OOCYTE DONOR HEIFERS FOR BOVINE NUCLEAR TRANSFER USING ADULT EAR SKIN FIBROBLASTS OF A BRAZILIAN CREOLE BREED – PRELIMINARY RESULTS.

Iguma, L.T.[1,2]; Pivato, I.[2,3]; Câmara, J.U.[2]; Damato, J.[2]; Pereira, D.C.[2]; Corrêa, G.A.[2]; Machado, G.M.[4]; Franco, M.M.[2]; Borges, J.R.J.[4]; Rumpf, R.[2]

[1]Department of Molecular Biology, University of Brasília, Brasília, DF, Brazil. [2]Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil. [3]CIDASC, Florianópolis, SC, Brazil. [4] Hospital of Veterinary, Faculty of Agronomy and Veterinary, University of Brasília, Brasília, DF, Brazil. rodolfo@cenargen.embrapa.br

The importance of cloning by adult somatic cell nuclear transfer (SCNT) in mammals is related to the great interest on its potential applications in several strategic areas (biomedicine, pharmacology, animal conservation, agriculture, fundamental science) and then, the invaluable impact for society. The present study was carried out to investigate the efficiency of nuclear transfer using recipient oocytes collected by ultrasound-guided follicle aspiration (ovum pick-up, OPU) from heifers stimulated with follicle-stimulating hormone (FSH) and with synchronized follicular wave. Donor cells were isolated from an ear skin biopsy of a 9-years-old Junqueira cow (*Bos taurus*), a specimen of endangered Brazilian Creole breed. For non-FSH stimulated group (T1), sixteen cyclic crossbred heifers had the ovaries aspirated by OPU. And for FSH-stimulated group (T2), 8 animals were selected from non-FSH group to be administered with 180 IU of FSH (Pluset, Calier, Spain), single dose, s.c., 72 h before OPU. In both groups seven sessions of OPU/SCNT was performed every four days. The oocytes from both treatments were in vitro matured for 19 to 21h and then, used as recipient cytoplasts in SCNT of adult cow ear skin fibroblasts (6 to 11 passages during in vitro culture). The data were statistically analyzed by qui-square test and $P<0.05$ were considered significantly different. For the control of activation procedures and culture system, a sample of oocytes from each manipulation was parthenogenetically activated and there were no significant differences observed either for cleavage (70.33% vs 78.31%) or blastocyst (53.38% vs 54.24%) rates between T1 and T2, respectively. The mean (±SE) number of follicles aspirated and oocytes collected per animal per OPU session was significantly lower ($P<0.01$) for T1 (7.7 ± 0.24 and 5.3 ± 0.29) than for T2 (11.2 ± 0.52 and 8.1 ± 0.50). But, the retrieval rate was similar for both treatments ($66.0 \pm 2.64\%$ and $71.5 \pm 2.93\%$, for T1 and T2, respectively). A total of 470 and 411 oocytes were recovered from T1 and T2, respectively. No significant differences ($P>0.05$) were observed in fusion (84.4% vs. 89.03%) and blastocyst (46.36% vs. 37.66%) rates between NT using non-FSH or FSH stimulated oocytes, respectively. However, cleavage rate was higher ($P=0.031$) for T1 (70.90%) than for T2 (57.14%). From T1, 46 embryos were transferred to 34 recipient cows and from T2, 36 embryos were introduced in 29 recipients. Ultrasonographic assessment showed no significant difference between T1 and T2 regarding initial pregnancy rate (~40 days; 61.76% vs 41.38%), 60 days of gestation (26.47% vs 17.24%) and 90 days of gestation (11.77% vs 10.35%). Some embryos from both treatments were separated for posterior gene expression analyses. These results suggest that FSH stimulation increased the number of oocytes retrieved by OPU. However, the administration of FSH in donor oocyte heifers with synchronized follicular wave did not influence the SCNT parameters evaluated in this study.

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OOCYTE TRANSFER INTO BUFFALO OVIDUCT OF FEMALES WITH SINGLE AND MULTIPLE OVULATIONS

Carvalho, N.A.T.[1,2]; Gimenes, L.U.[1]; Sá Filho, M.F.[1]; Vannucci, F.S.[1]; Reichert, R.H.[2]; Carvalho, J.B.P.[3]; Souza, R.J.[4]; D'Angelo, M.[4]; Chiari, J.R.[5]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ-USP-SP, 05508-000; [2]APTA-Vale do Ribeira; [3]APTA-Pindamonhangaba;[4]Instituto Biológico-SP; [5]SAMVET, Morrinhos-GO. nelcio@usp.br

The aim of the present study was to evaluate bovine and buffalo oocyte transport through genital system of bovine females with single and multiple ovulations. Twelve buffalo females were assigned in four groups (2x2 factorial). All females received 2mg of estradiol benzoate i.m. (Estrogin®, Farmavet, Brasil) and a progesterone intravaginal releasing device (DIB®, Syntex, Argentina) on random day of estrous cycle (Day 0). On Day 4, the animals of G2 and G4 groups (multiple ovulations), received 2500UI of eCG i.m. (Novormon®, Syntex, Argentina) On day 8, DIB® was removed plus 0.150mg of PGF2α i.m. (Prolise®, Arsa, Argentina). The females of G1 and G3 groups (single ovulation) received 400UI of eCG (Novormon®) on Day 8. On Day 9, GnRH i.m. (Gestran Plus®; 50µg de Lecirelina, Arsa, Argentina) was injected and, after 12 and 24 h, the females were inseminated with buffalo (G1 and G2) and bovine (G3 e G4) semen. On Day 10, all females were submitted to a right flank laparotomy and to exposure of oviduct ipsilateral to ovulation (G1 e G3) or ipsilateral to side that had more ovulations (G2 e G4). In G1 and G2 groups, 10 buffalo oocytes submitted to maturation were inserted in the infundibulum while in G3 and G4 groups the same process was performed with 10 bovine oocytes. The recovery of embryo structures was performed by transcervical uterine flushing on Day 15 (G1 and G2) and on Day 16 (G3 and G4), using 1L of DPBS (Cultilab®). The ovulatory rates to G1, G2, G3 e G4 were, respectively: 100,0±0,0%, 33,3±16,7%, 100,0±0,0%, 36,9±10,2%. No structures were recovered in any group. This result can be occurred due to embryo recovery rate compromising in superstimulated buffalo females (Baruselli et al., 2000; Theriogenology, v.53, p.491) or due to oviduct manipulation affecting the gamete transport.

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ORAL PRESENTATION

EFFECTS OF THE MOMENT OF $PGF_{2\alpha}$ IN FIXED TIME EMBRYO TRANSFER PROTOCOL ON SYNCHRONIZATION AND CONCEPTION RATES IN IVF FRESH EMBRYO RECIPIENTS

Sá Filho, O.G.[1]; Vasconcelos, J.L.M.[1]; Santos, R.M.[1]; Oba, E.[2]; Perez, G.C.[1]; Araújo, D.B.[1]

[1]Departamento de Produção e Exploração Animal – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. [2] Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. vasconcelos@fca.unesp.br

The present trial tested if anticipation of PGF2α injection in a fixed time embryo transfer (FTET) protocol alters ovulatory follicle diameter, synchronization rate, CL diameter, serum P4 concentration, conception and pregnancy rates in IVF fresh embryo recipients. Cycling crossbred heifers (n=153), BCS 3.3±0.17 (1-5 scale), were divided in 4 groups: G1 (n=42) Estradiol Benzoate (EB; Estrogin, Farmavet, Brazil, 2mg i.m.) + new CIDR - 7d - PGF2α (Lutalyse, Pfizer, Brazil, 25mg i.m.) - 2d - CIDR withdrawal - 1d - EB (0.8mg) - 8d - FTET; G2 (n=41) similar to G1, but with a 9 days previously used CIDR; G3 (n=35) EB (2mg) + new CIDR - 9d - CIDR removal + PGF2α - 1d - EB (0.8mg) - 8d - FTET; G4 (n=35) similar to G3, but with a 9 days previously used CIDR. All heifers with CL at ET day received fresh IVF embryos (grade 1 or 2) transferred by a trained technician. Ovulatory follicle diameter at moment of CIDR removal, CL diameter and serum P4 at FTET were analyzed by GLM; synchronization (presence of CL at FTET), conception and pregnancy rates were analyzed by logistic regression. There was no effect of treatment on ovulatory follicle diameter (10.58±0.56mm; 11.05±0.63mm; 11.05±0.58mm; 10.67±0.56mm; P=0.49), synchronization rate (83.33%; 65.85%; 82.86%; 80.00%; P=0.28), CL diameter (18.93±0.66mm; 19.72±0.71mm; 19.20±0.66mm; 18.85±0.67mm; P=0.40) and serum P4 (2.87±0.33ng/mL; 2.28±0.33ng/mL; 2.69±0.34ng/mL; 2.05±0.35ng/mL; P=0.77) for groups 1, 2, 3 and 4, respectively. Treatment tended to affect conception (38.23%; 30.77%; 13.79%; 25.00%; P=0.12) and pregnancy rates (31.71%; 20.00%; 11.43%; 20.00%; P=0.14). Recipients receiving PGF2α on day 7 tended to have greater serum P4 concentrations than recipients receiving on day 9 (G1+G3: 2.78±0.24ng/mL; G2+G4: 2.15±0.24ng/mL; P=0.06). Regardless of treatment, ovulatory follicle diameter affected ovulation rate (P<0.001) and CL diameter (P<0.001). Serum P4 concentration were affected by CL diameter (P<0.05) and tended to be affected by ovulatory follicle diameter (P=0.07). Conception and pregnancy rates were affected by serum P4 concentration (P<0.01) and CL diameter (P<0.01). These data suggest that anticipation of PGF2α injection in a FTET protocol did not affected ovulatory follicle diameter, synchronization rate and CL diameter, but increased serum P4 concentrations, which improve conception.

EFFECT OF PROGESTERONE SUPPLEMENTATION ON SUPEROVULATORY RESPONSE IN NELORE COWS (*Bos indicus*) DURING THE FIRST-FOLLICULAR WAVE

Nasser, L.F.T.[1]; Bó, G.A.[2]; Reis, E.L.[1]; Menegati, J.A.[1]; Marques, M.O.[1]; Gimenes, L.U.[1]; Sá Filho, M.F.[1]; Mapletoft, R.J.[3]; Baruselli, P.S. [1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo/ SP, Brazil. [2] Instituto de Reproduccion Animal Córdoba (IRAC), Córdoba, Argentina. [3]University of Saskatchewan, SK, Canadá. vasconcelos@fca.unesp.br

A total of twenty Nelore cows (n=20) were homogeneously assigned in two experimental groups (WaveOne and WaveOne+P4). The females were pre-synchronized with 2mg of estradiol benzoate plus 50mg of progesterone (P4) i.m. (Index Farmacêutica, Brazil) and intravaginal progesterone releasing device (P4; CIDR, Pfzier, Brazil) on Day 0. On Day 5, PGF2α (Preloban, Intervet, Brazil) were administered. On Day 9, device was withdraw and 12.5mg of LHp (Lutropin, Vetrepharm, Canadá) were administered 24 hours later. The superstimulation was induced by 133mg of FSHp (Folltropin, Vetrepharm, Canadá), subdivided in ten equal doses every 12 hours from Day 11 to Day 15. On Day 15, the females received two doses of PGF2α (morning and afternoon) and 25mg of LHp were administered 24 hours later. The WaveOne+P4 Group received the same treatment, but was supplemented with P4 during the FSH treatment. The cows were inseminated in the morning and in the afternoon on Day 17 and the embryo recovery was performed on Day 23. The data were analyzed by Anova and Chi-square tests. The results for WaveOne vs. WaveOne+P4 Groups were, respectively: number of follicles ≥8mm at 2nd LH (19.9 ± 2.2 vs. 14.5 ± 3.5; $p>0.05$), number of CLs at embryo recovery (5.36 ± 1.7^a vs. 9.1 ± 2.2^b; $p=0.06$), ovulatory rates (76.9 ± 7.6 vs. $69.2\pm8.4\%$; $p>0.05$), embryo recovery rates (31.0 ± 10.7 vs. $54.8\pm11.1\%$; $p>0.05$), total of structures (4.0 ± 1.5 vs. 4.7 ± 1.1; $p>0.05$), transferable embryos (1.3 ± 1.3^a vs. 3.9 ± 1.1^b; $p<0.05$), embryos suitable for freezing (1.2 ± 1.2^a vs. 3.0 ± 0.9^b; $p<0.05$), unfertilized structures (2.5 ± 0.75^a vs. 0.2 ± 0.2^b; $p<0.05$) and plasmatic progesterone concentrations during the treatment (1.32 ± 0.16^a vs. 3.5 ± 0.3^b ng/ml; $p<0.05$). The results suggest that is possible to use first-wave for superovulation in Nelore cows if a P4 supplementation is done during superstimulation treatment.

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EFFECT OF SUPEROVULATORY TREATMENT OF MARES WITH EQUINE PITUITARY EXTRACT ON OOCYTE TRANSPORT TO THE OVIDUCT (Preliminary Results)

Carmo, M.T.[1]; Losinno, L.[2]; Aguilar, J.J.[2]; Araujo, G.H.M.[1]; Alvarenga, M.A.[1]

[1]Department of Animal Reproduction and Veterinary Radiology, Faculty of Veterinary, FMVZ – UNESP – Botucatu, SP, Brazil [2]University of Rio Cuarto, Rio Cuarto – Argentina.
mtc.vet@uol.com.br

There is more them 30 years of studies looking for a ideal protocol to induce multiple ovulations in mares. Better superovulatory response have being reported with new protocols using Equine Pituitary Extract (EPE), with a still low embryo recovery rate. The aim of the present study was to evaluated if the low embryo recovery rate observed in superovulated (SO) mares is related with disturbs during ovulation and /or transport of the oocytes to oviduct. This on going experiment was developed in a horse slaughter house Argentina with the collaboration of University of Rio Cuarto-Argentina. A total of eighteen mares aging between 3 -12 years were used , 12 EPE treated and 6 control (saline treated). Mares were evaluated daily by ultra-sound to control follicular growth and ovulation. The EPE treatment (25mg twice daily) started on day 7 after ovulation and was discontinued when a the majority of the follicles reached 35 mm and 3000 UI of HCG was administered. Mares were slaughter 12 to 24 hours after first ovulation was detected. The oviducts were transported to the laboratory 20 to 30 minutes after mares slaughter and flushed with DPBS. Oocytes were searched in a stereomicroscopy and morphologically classified. Data were analyzed by ANOVA and Chi-square test. It was observed that SO mares had a higher ($p<0,05$) number of ovulations per cycle ($4,417 \pm 2,906$ / $1,167 \pm 0,408$) and a higher number of oocytes recovered per mare ($2,67 \pm 1,624$ / $1,0 \pm 0,632$). No statistical difference ($p>0.05$) was observed on the percentage of viable oocytes recovered per ovulation in SO mares (60%) and non superovulated mares (85%). The decreased observed in the percentage of viable oocytes recovered by ovulation is similar with the reported in the embryo recovery rates of SO mares. The partial results of this experiment indicated that disturbs on transport of oocyes to oviduct can be involved on low embryo recovery rates of SO mares. Studies are in progress to determined the factors involved on this fail and also to evaluated the maturation status of the recovered oocytes.

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TREATMENT OF MANGALARGA MARCHADOR MARES WITH PROGESTERONE (P4) AND ALTRENOGEST AIMING THE UTILIZATION AS EMBRYO RECIPIENTS ON DAY TWO AFTER OVULATION

Caiado, J.R.C.[1]; Fonseca, F.A.[1]; Silva, J.F.S.[1]; Fontes, R.S.[1]

[1]Universidade Estadual do Norte Fluminense-Centro de Ciências e Tecnologias Agropecuárias. Avenida Alberto Lamego-100. Campos dos Goytacazes-RJ. jrccaiado@hotmail.com

During the reproductive season of 2001 and 2002, 43 mares of mangalarga marchador breed, recipients of embryos, were treated randomly with intra-muscular application of 200 mg/day of oily progesterone (P4). Another group of 41 mares of the same breed, were treated with 0,044 mg/kg/day of altrenogest, randomly via oral, with the objective of precocity use in embryo transfer. Both treatments were performed between day 0 (D 0) and day 5 (D5) after ovulation. The recipients were evaluated on D2 and the ones considered in good or excellent conditions were inovulated. Two groups of 85 mares of the same breed were used randomly as control and did not receive any kind of progestagen. They were also evaluated and, when considered appropriate, inovulated on D2 or D5. The groups D2/P4 and D5 without any progestagen treatment presented a significantly greater ($P<0.05$) percentage of recipients considered excellent or good (appropriate) during the evaluation, respectively 55 (64.71%) and 25 (65.12%), evaluated by qui-square method. In the group D2 with altrenogest, 20 mares were considered appropriate for inovulation and 21 were considered not appropriate, numbers were considered statistically not different. The group D2 without progestagen showed a significantly greater percentage of mares considered not appropriate (65 ou 76,47%). With respect to the pregnancy rate after inovulation, the groups D2/P4 and D5 without progestagen presented numbers significantly ($P<0.05$) greater of pregnant recipients, respectively 72.72%(16-22) and 76.36%(42-55), than non-pregnant. The group D2 with altrenogest presented a pregnancy rate of 52.38% (11-21) considered not significant greater than the rate of non pregnancy ($p>0.05$). The group D2 without progestagen presented a significantly smaller number pregnant recipients (30% - 6 from 20). These data allow us to concluded that the treatment of recipient mares with P4 from D0 to D5, is a good alternative to anticipated to D2 the utilization of recipient mares with similar results to that observed in recipient mares on D5.

GENE EXPRESSION AT MATERNAL-ZYGOTIC TRANSITION IN *IN VITRO* FERTILIZED AND SOMATIC CELL NUCLEAR TRANSFER BOVINE EMBRYOS

Camargo, L.S.A.[1]; Powell, A.M.[2]; Viana, J.H.M.[1]; Sá, W.F.[1]; Ferreira, A.M.[1]; Ramos, A.A.[3]; Wall, R.J.[2]

[1]Embrapa Dairy Cattle, Juiz de Fora, MG, Brazil. 36038-330. [2]Biotechnology and Germplasm Laboratory, BARC, USDA, Maryland, EUA. 20705. [3]Epamig, Juiz de Fora, MG, Brazil. 36045-560. camargo@cnpgl.embrapa.br

During early development, the embryonic genome undergoes reprogramming, activating the expression of some genes while repressing others. It has been suggested that nuclear transfer may cause alterations in gene expression during this period. The aim of this study was to compare the gene expression during maternal-zygotic transition (MZT) between *in vitro* fertilized (IVF) and somatic cell nuclear transfer (SCNT) bovine embryos. SCNT embryos were produced with serum-starved fibroblast cell obtained from three months-old fetuses muscle. IVF zygotes and SCNT embryos were cultured in G1.1 medium without serum for 70-74h under 5% CO_2, 5% O_2 and 90% N_2. Eight to sixteen-cells stage embryos were frozen and stored in N_2 liquid until RNA extraction. Extracted RNA was amplified and all genes were analyzed in individual embryos. Twenty-three IVF and 24 SCNT embryos were evaluated. The genes studied encode GLUT-5 (glucose transporter-5), Na^+/K^+ ATPase $\alpha 1$ and LDHA (lactate dehydrogenase isoenzyme A) proteins, initially expressed at the start of MZT. Expression of OCT-4, specific for totipotent cells, and collagen VI$\alpha 1$, specific for fibroblast, were also analyzed. Relative quantification was performed using real time PCR, in duplicate, and the results were normalized against expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as internal reference gene. Statistical analysis was performed by ANOVA. No difference in relative abundance ($P>0.05$) was observed between IVF and SCNT embryos for all genes evaluated. Collagen VI$\alpha 1$ expression was not detected in IVF and SCNT embryos but was readily detected in fibroblast cells used as nucleus donors. These results show that SCNT embryos were able to initiate expression of the genes analyzed during early embryo development, including the gene associated with totipotency, while repressing the expression of a somatic gene. This suggests that nuclear transfer does not alter expression of those genes in embryos during MZT, when compared to IVF embryos.

IN VITRO EFFICIENCY OF TWO DIFFERENT TECHNICAL APPROACHS TO PRODUCE BOVINE CLONED EMBRYOS

Feltrin, C.; Forell, F.; dos Santos, L.C.; Vieira, A.D.; Rodrigues, J.L.

Laboratório de Embriologia e Biotécnicas de Reprodução, FAVET, UFRGS
CP 15004, 91501-970, Porto Alegre, RS, Brasil – cristianofeltrin@terra.com.br

The objective of this experiment was to compare the in vitro efficiency of two technical approachs, the Handmade Cloning (HMC), described by Vajta (2003) and modified by Bertolini (personal communication) and the micromanipulation procedure (CC) to produce in vitro bovine clone embryos. Six hundred bovine oocytes, in three replicates, were selected after been collected by slicing from bovine ovaries obtained from the slaughterhouse. To undergo IVM the oocytes were exposed during 19 hours to TCM199 medium supplemented with 10% ECS, 0,5 g/mL FSH and 0,03UI hCG and maintained at 39ºC in humid atmosphere containing 5% CO_2 in air. After the removal of the cumulus oophorus cells, the matured oocytes (234 HMC group and 219 CC group) were transferred to under mineral drops of a modified SOF medium supplemented with hepes (25mM) and 0,4% of BSA, maintained at 37ºC. To use the HMC approach, first the oocytes were exposed to a enzyme solution to promote the zona pelucida digestion. After that the oocytes were manually divided in two halfs and reconstituted with the somatic cells with the aid of one phytohemogglutinin (550 µg/mL) solution. Finally, the reconstructed structures were fused in one fusion chamber. On the other hand in the group of oocytes manipulated by the CC apporach, the somatic cell enucleation and electrofusion with the oocyte were performed using a micromanipulator and electrodes. The fusioned embryos in both experimental approachs were activated by exposition to 5µM of ionomycin (during 5 min) solution and to 2mM of 6-DMAP (during 3h and 30min) solution. The embryos were then transferred to a modified SOF medium supplemented with aminoacids and 10% of ECS and cultured at 39ºC, in humid atmosphere containing 5% CO_2, 5% O_2 and 90% N_2. The cleavage and blastocyst development rates were evaluated 48 hours and 168 hours after the in vitro culture has been started. Using the HMC approach from 234 matured oocytes, 220 (94%) could be successfully manipulated and 98 (44,5%) structures were reconstructed. The cleavage rate was 74,4% (73/98) and the rate of embryonic development to the blastocyst stage was 18.3% (18/98). On the other hand the use of CC allows from 219 maturated oocytes the micromanipulation of 105 (48%) and 37 (35.22%) reconstructions. The cleavage rate was 56.7% (21/37) and the rate of embryonic development to the blastocyst stage was 13.5% (5/37). The HMC allowed the manipulation and reconstruction of a larger number of structures ($p<0.01$) and the cleavage rate was significantly greater ($p<0.05$) than the observed with the use of CC. On the other hand, the rate of embryonic development to the blastocyst stage was similar in the two embryo clone producing approachs used in this experiment.

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INFLUENCE OF NELORE BLOOD ON THE *IN VIVO* PRODUCTION OF OÓCYTES

Rubin, K.C.P.[1]; Pontes, J.H.F.[2]; Nonato Jr., I.[2]; Ereno Jr., J.C.[2]; Pansard, H.[3]; Seneda, M.M.[1]

[1]Departamento de Clínicas Veterinárias, CCA, Universidade Estadual de Londrina, PR, 86051-990. karinac8@aol.com. [2]In Vitro Brasil–Central de Biotecnologia da Reprodução–Mogi Mirim. [3]Fazenda Santa Gabriela - Uraí – PR.

Nowadays, Brazil has a bovine herd with 180 milions animals, from which 80% are *Bos indicus* or crossbreeding with *Bos indicus* and from this, 95 milions are from Nelore breed (Nogueira, 2004). Based on this quotation, the present paper has as its objective to analyse the influence of the Nelore breed blood percentage on the ovocytes production. It was analysed the oocytes production of 172 cows, 41 from Aquitanica, 44 from Nelore and 87 from Canchim breed. This animals were submitted to an oocytes recovery by follicular aspiration in a random moment of estrous cycle. Aquitanica breed animals obtained an average of 3.7 aspirated oocytes for each aspiration session, Canchim animals obtained a 13.6 average and animals from Nelore breed 18.4. There were significant differences ($p<0.05$) between each breed average of aspirated oocytes. It becomes evident that rising the percentage of Nelore blood on the racial constitution, there are also increase the ovocytes production, since the higher average of aspirated oocytes was found on Nelore animals, the second higher average was found on Canchim breed (5/8 Charoles blood and 3/8 Nelore blood), and so the lowest average was found on the animals from Aquitanica breed, that has no Nelore blood on this racial constitution. Although this datas are in accordance to other works from literature, we emphasize that this results are only initial and better evaluation of the results will be possible with the work continuity.

EFFECT OF EXOGEN PROGESTERONE IN OOCYTE DONORS COWS ON FOLLICLE DEVELOPMENT AND *IN VITRO* EMBRYO PRODUCTION

Pfeifer, L.F.[1]; Schneider, A.[1]; Castilho, E.M.[1]; Luz, E.M.[1]; Ataíde, P.F.[1]; Dionello, N.J.L.[2]; Pivato, I.[3]; Rumpf, R.[4]; Corrêa, M.N.[1]

[1]Faculty of Veterinary - NUPEEC - Federal University of Pelotas, 96010 900, Pelotas-RS. [2] Faculty of Agronomy - Federal University of Pelotas, 96010 900, Pelotas-RS. [3] CIDASC- Integrated Company of Agrary Development of Santa Catarina, 89130 000, Indaial-SC, Brazil. [4] EMBRAPA – National Center of Genetic Resources and Biotechnology, 70770 901, Brasília-DF, Brazil.

lpfeifer@ufpel.edu.br

The follicular punction (FP) technique makes possible improve the use of oocytes that physiologically will undergo atresia, increasing the reproductive efficacy and the genetic merit of cattle, showing viability even on females with reproductive disorders. The aim of this work was to evaluate the follicles number able to FP, the quantity and quality of oocytes and the *in vitro* production of blastocysts from donors submitted to different treatments with progesterone. For this experiment 15 crowsbreed cows were used. Before the beginning of the experiment, all cows received a CIDR®(intravaginal progesterone releaser dispositive) for 8 days. Two days before CIDR® withdraw, all cows received 0.150 mg of D-cloprostenol. In the day of CIDR® withdraw all cows were submitted to FP. After this first FP the cows were randomically divided in three different groups and submitted to FP each 4 days in a total of 6 sections. G1: the animals received a used CIDR®; G2: animals that received 2 new dispositives, and G3: animals that weren't submitted to CIDR® treatment. The changes of CIDR® in G1 and G2 were performed each 8 days, like the application of 0.150 mg of D-cloprostenol. The results show an increase ($p<0.01$) on the mean of small follicles, with 5mm of diameter, in G1 (9.44) and G2 (9.44) compared to G3 (6.12). There was a difference ($p<0.05$) in the recuperation rate only between G2 (68.77%) and G3 (47.19%) and an increase ($p<0.01$) in the oocytes/cow/punction collected of G1 (6.56) and G2 (6.96) compared to G3 (3.36). Also was observed a difference ($p<0.05$) in the mean of oocytes with quality I and II among the G1 and G3, but no difference ($p>0.05$) was observed for G1 e G2 and G3 (13 for G1, 5.6 for G2 and 2.6 for G3). The cleavage rate of oocytes of qualities I and II of G1 (80%) was higher ($p<0.05$) than G2 (75%) and G3 (61.5%). The blastocyst rate, coming from oocytes of I and II qualities was higher ($p<0.05$), in G1 (46.15%) compared to G3 (15.38%), however, there was no difference between G1 and G2 (32.14%) and between G2 and G3. There was no difference ($p>0.05$) in the total blastocyst rate between groups: 28.20%, 16% and 15% for G1, G2 and G3, respectively. The results show that the cows treated with progesterone implants have an increase of oocytes quantity, as well as follicles able to punction. The oocytes of qualities I and II recovered and the blastocysts rate production coming from these oocytes was higher when donors were submitted to treatment with only one reused CIDR® in relation to cows that were not treated, furthermore, the cleavage rate of that oocytes was higher. The total cleavage rate was higher when the cows were submitted to treatments with progesterone, however it was no difference in the rate of total blastocysts production between groups.

COMPARISON OF PREGNANCY LOSSES IN EMBRYO RECIPIENTS PREGNANT OF *Bos indicus* EMBRYOS PRODUCED *IN VIVO* OR *IN VITRO*

Borsato, E.A.[1]; Ludwig Jr, H.E.[1]; Silva, D.R.M.[1]; Barreiros, T.R.R.[2] ; Seneda, M.M.[3]

[1] Embriogen, Campo Mourão, PR, [2] Faculdades Integrado de Campo Mourão, PR; [3]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051- 990 thalesrigo@grupointegrado.br

In order to increase reproduction efficiency, there are a lot of researches about differences of pregnancy rates between embryo period and fetus period of pregnant female cattle. The objective of this study was to compare pregnancy losses of transferred recipients heifers with embryos produced *in vivo* and *in vitro*. One hundred and sixty five heifers in estrus of *Bos taurus* x *Bos indicus* on pasture, in Campo Mourão region, Paraná. These animals were selected after a previous treatment for embryo transfer at fixed time (ETFT). In this treatment in a random stage of estrus cycle, was introduced an intravaginal device of 1.9 g of P4 (CIDR, Pfizer, Brazil) at the same time as an application of 2mg IM of EB (Estrogin, Farmavet, Brazil). On the eighth day, those devices were removed and animals received 500 μg IM of cloprostenol (Sincrocio, Ouro fino, Brazil). After twenty-four hours all animals received one application of 1 mg IM of EB. Ultrasonography (Aloka, SSD 500, and 5 MHz) identification and valuation of CL were carried out 16 days after treatment beginning. In the first group (G-1, n=59), after ultrasonography, heifers were transfered with embryos produced by superovulation and transcervical recovery. Pregnancy diagnosis was done on 30 and 60 days by ultrasonography method. Pregnancy losses were calculated based on pregnancy rates differences established on 30 and 60 days. This information was analyzed in agreement with the test of the corrected Qui-square of Yates. No significant differences were detected on rates pregnancy between the groups G-1 and G-2, and consequently resulting, respectively, in 57.6% (34/59) and 52.1% (50/96) at 30 days of pregnancy and in 49.1% (29/59) and 40.6% (39/96) (P>0.05) at 60 days. In the G-1 pregnancy losses were 8.5% (5/50) and in the G-2 losses were 11.4% (11/96) (P>0.05).Therefore, did not observed difference between pregnancy rates and pregnancy losses of receptor cows subject to ETFT with embryos produced *in vitro* and *in vivo*. This information shows that independent of the embryos production method, it is necessary the development of methods which try to increase pregnancy rates on embryo transfer.

INVOLUTION OF THE YOLK SAC IN IVF AND NATURAL STUD BOVINE EMBRYOS (10 TO 70 DAYS OF GESTATION)

Assis Neto, A.C.[1,2]; Silva, T.C.B.[1]; Verechia, F.T.V.[1]; Ambrósio, C.E.[2]; Almeida, A.B.; Meirelles, F.V.[3]; Garcia, J.M.[4]; Miglino, M.A.[2].

[1] Faculty of Zootecny -Dracena /UNESP, Dracena-SP, 17900-000, Brazil. [2] Department of Surgery – FMVZ/USP, São Paulo-SP, Brasil. [3] FZEA/USP, Pirassununga-SP, Brasil. [4] Department of Animal Reproduction and Preventive Veterinary Medicine – FMVZ/UNESP, Jaboticabal, Brasil.

antonioassis@dracena.unesp.br

The yolk sac involution and degeneration is very early and none vestige is found in the end of gestation. Considering that the yolk sac is very important to embryonic circulation and metabolic transmission, the objective of this study was to characterized morphometrically the involution of the yolk sac originated after IVF technique and natural stud. Fifty nine embryos (10 to 50 days of gestation), nine fetus in initial period (60 to 70 days of gestation) obtained after natural fertilization and six originated after IVF technique (35 to 46 days of gestation) were used. The embryos and fetuses originated of the natural stud were grouped by mensuration of the nucal distance to last sacral vertebra and histologic characteristics of embryonic development. The yolk sac is constituted to the 10-20 days by a compacted central area and two free elongated extremities. With the evolution of the gestation this structure was decreasing it size and to leave of 40 to 50 days of gestation, your total size decrease considerable in relation to anterior periods. The involution of the yolk sac was established in the fetuses with 60 to 70 days of gestation. The average of the Crown-Rump, the weight of the embryo an the gestational sac grown up lineally with the evolution of the gestation. In these two last parameters the growing was faster in the 40-50 days of gestation. The yolk sac of the IVF embryos had a lesser length (1,07±0,55 cm) when compared with the natural stud group (5,53 ±3,14 cm) in a 30-40 days of gestation. We can concluded that the yolk sac of natural stud embryos had grown until the 40 days of gestation; in a 70 days of gestation the remained yolk sac was present and in the IVF embryos was less developed in 30 to 40 days of gestation.

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Abstracts - Session A

EFFECT OF DIFFERENT SUPEROVULATION PROTOCOLS ON EMBRYO PRODUCTION IN MICE (*Mus musculus*) FROM SWISS LINEAGE

Wohlers, S.[1]; Pinto, I.S.B.[1]; Arashiro, E.K.N.[2]; Viana, J.H.M.[1,2]

[1] Centro de Ensino Superior de Juiz de Fora, Juiz de Fora, MG 36016-000, Brazil
[2] Embrapa Gado de Leite, Juiz de Fora, MG - 36038-330, Brazil
jhmviana@ig.com.br

The negative feedback produced by dominant follicles inhibin shows an important effect on FSH regulation. The immunization against inhibin has been used to increase natural ovulation rates in different species, and also in association with conventional superovulation protocols. The aim of this study was to evaluate different superovulation protocols on mice (*Mus musculus*) ovulation rate and embryo yield.. Female mice (N=32) were randomly distributed among four treatments: control (T1); conventional superovulation (T2); active immunization against bovine inhibin (T3); active immunization associated with superovulation (T4). Superovulation were induced by intraperitoneal administration of 10 U.I. of eCG, followed by 10 U.I. of LH after 48h. Steroid-free follicular fluid, obtained from follicles larger than 9mm and in growing phase, conjugated with Freund´s adjuvant, was used as immunogen. Two booster injections were given seven days apart. The average number of ovulations per animal was 10.90±1.16, with the recovery of 7.39±0.92 total structures, from which 5.18±0.74 were classified as viable embryos (67.0%). Immunization against inhibin did not affect the number of total structures or viable embryos recovered, when compared to the control group (5.67±0.73 and 3.12±1.06 vs. 4.17±0.96 and 2.33±1.35 on groups T3 and T1; respectively; $P>0.05$), but enhanced the effect of conventional treatment on the number of ovulations and total structures recovered (20.50±2.81 vs. 9.00±1.84 and 12.60±2.35 vs. 9.40±0.96 on groups T4 and T2, respectively, $P<0.05$). However, T4 showed a lower percentage of viable embryos than T2 (63.5 vs. 74.5%), resulting in a similar number of viable embryos recovered (5.71±1.96 vs. 7.00±1.35; $P>0.05$). These results suggest that active immunization against bovine inhibin affect superovulation response in mice, and this effect can be used as an experimental model for the study of inhibin actions in ovarian physiology.

INCIDENCE OF MULTIPLE OVULATIONS IN THE CAMPOLINA BREED AND ITS INFLUENCE ON EMBRYO RECOVERY

Greco, G.M.[1]; Oliveira, G.D.M.[2]; Pinna, A.E.[2]; Pinho, T.G.[3]; Queiroz, F.J.R.[2]

[1] Graduation Student e Research Student- CNPq – Dep. of Physiopathology of Reproduction – UFF, Niterói-RJ, 24230-340, Brazil [2] Equus Veterinary Clinic [3] Dep. of Physiopathology of Reproduction – UFF. gabvet@ig.com.br

Multiple ovulations are desirable in embryo transfer programs, for they can increase the embryo recovery by one or more embryos in each uterine trans-cervical flush perfomed, leading to better results. Horses are classified as mono-ovulatory animals; however, the incidence of double ovulations in the species is variable, depending on the mare´s breed, her family line (MCKINNON, A.O., *Equine Reproduction*, p.125, 1993), and age (MOREL, M.C.G.D., *Animal Reproduction Science*, 66: p.59-70, 2001). Double ovulations are a common occurence, having a reported incidence that ranges from 4% to 50% (MCKINNON, A.O., *Equine Reproduction*, p.125, 1993; CARMO et al., *ICAR 2004 Abstracts*, Vol.2, p.406, 2004), which makes its study on different populations necessary. The present experiment's objective was to identify, in the Campolina breed, the incidence of these different ovulatory patterns, as for their influence on embryo recovery. 109 mares had their estrous cycles recorded through rectal palpation and ultrasonographic evaluation during the breeding season of 2002 – 2003, in a total of 364 cycles. These evaluations began when the first signs of estrous behavior were shown or, in those mares which were already in the embryo transfer process, five days after an uterine flush was performed, until 24 hours were passed after the first ovulation. Artificial inseminations were made with semen of fertile stallions. The obtained results were analyzed by the Chi-Square test. Multiple ovulations occurred in 30.8% (112/364) of the cycles; 69.2% of the cycles (252/364) leaded to simple ovulations, 15.4% (56/364) to unilateral double ovulations and 13.2% (48/364) to bilateral double ovulations. The incidence of triple ovulations was low, 1.9% (7/364), as of quadruple ovulations 0.3% (1/364). Most of the simple ovulations happened on the left ovary (52.8% (133/252) against 47.2% (119/252) on the right ovary; but this fact did not have any statistical relevance. Out of a total of 364 flushes, 249 (68.4%) ended on the recovery of at least one embryo; the overall recovery rate was 81.9%. Bilateral double ovulations resulted in a higher embryo recovery rate ($p < 0.05$) when compared to unilateral double ovulations (137.5% against 101.8%, respectively). Moreover, the number of flushes in which two embryos were recovered was higher ($p < 0.05$) in those cycles where double bilateral ovulations occurred (54.2% against 33.9%). Through the analysis of these results, we can conclude that the Campolina breed has a high incidence of multiple (30.8%) and double (28.6%) ovulations, since, in the horse species, it averages around 16% (BLANCHARD, T.L., *Manual of Equine Reproduction*, p.11, 2002). We can detect that double bilateral ovulations result in higher embryo recovery rates, giving evidence, once again, that the fertility is affected when multiple ovulations within the same ovary occur. Possibly, the incidence of triple and quadruple ovulations detected could have been higher, if the evaluations persisted for more than 24 hours after the first ovulation.

CORRELATION AMONG THE DIAMETER OF THE CORPUS LUTEUM FORMED FROM SINGLE AND DOUBLE OVULATIONS AND THE PLASMATIC LEVEL OF PROGESTERONE IN JENNIES

Conceição, J.C.[1]; Araujo, G.H.M.[1]; Oliveira, J.V.[2]; Oba, E.[1]; Carvalho, L.R.[3]; Meira, C.[1]

[1]Department of Animal Reproduction and Veterinary Radiology – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. [2]Pólo Regional de Desenvolvimento Tecnológico da Alta Mogiana – E.E.Z., Colina-SP, Brasil. , [3]Statistical Department - IB/UNESP. julianazuppi@bol.com.br

The present study aimed to compare the corpora luteum (CL) with diameters formed from single and double ovulations and to correlate this event the plasma progesterone concentrations (P4). Although the amount of reports about development CL in mares, there is just a few or none research involving asinine specie in this area. Seven Jennies from the Marchador Brasileiro breed, in full reproductive activity were divided in two groups: Group 1, Jennies with a single ovulation (n=4); Group 2, Jennies with synchronic double ovulations [synchronic=time interval < 24h between the two ovulations (n=3)]. The ovaries were examined by rectal ultrasonography and blood sample were collected daily, during the estrus cycle. The blood samples were centrifuged and the plasma stored at -20° for P4 measurements by Radioimmunoassay. The comparison of the means (diameters and days) between the two groups was performed by ANOVA, with daily P4 concentrations and CL diameters submitted to t-test at, both at 5% error probability. Linear regression was used to analyze the P4 concentration vs CL diameter. On the first day after ovulation, the mean and standard deviation of the CL diameter formed by single ovulations was 26.22 ± 4.44 and for the double ovulations 22.08 ± 2.67mm, no differences were observed ($p>0.05$). The follicles that leaded to double ovulations were smaller than the single ovulations follicles: 34.63 ± 1.65 and 39.44 ± 2.71mm respectively, however with no difference ($p>0.05$). Corpora luteum diameter means between the two groups were compared day by day and a numerical difference was verified being maintained until the last CL observations (Group 1: 12.25 ± 2.5 days and Group 2: 10.33 ± 1.75 days, $p>0.05$). The formed CL diameters were: of the single and double ovulatory follicles respectively ($p>0.05$), like other observations in mares raging from 50 to 75% (Ginther, 1992; *Reproductive biology of the mare, 2nd ed., Equiservices, Cross Plaine*). There was no difference on P4 concentrations between the two groups, despite of the two CLs observed in each Jennies from G2, witch always presented a higher P4 concentrations. This data are in agreement with the reports in mares, where the presence of two CLs did not lead to P4 concentrations twice higher than the values forms in mares presenting one CL (Ginther, 1992). In addition to the observations previously described by Meira *et al.* (*Biol. Reprod. Mono. 1, p.403-10 1995*). Progesterone concentrations were higher from day 6 to 14 after ovulations in both groups, being the means and standard error from those days 26.05 ± 1.21 and 30.23 ± 1.05 ng/ml for G1 and G2 respectively. These values are higher than the normally values found in mares. The luteolysis occurred by day 17 (P4<2 ng/ml) in both groups. Were observed negative correlation among P4 concentration and CL diameter to single ovulation (R= -0,86) as double ovualtion (R= -0,73). From this data was concluded that the Jennie physiology has some peculiarities that deserves more detailed studies.

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EFFECT OF eFSH® TREATMENT ON LUTEAL FUNCTION IN SUPEROVULATED TRANSITIONAL MARES

Peres, K.R.[1]; Fernandes, C.B.[2]; Oba, E.[2]; Alvarenga, M.A.[2]; Landim-Alvarenga, F.C.[2]

[1] Dep. of Animal Reproduction Animal – FMVZ/USP –São Paulo, 05508-900, Brazil [2] Dep. of Animal Reproduction and Veterinary Radiology - FMVZ/UNESP – Botucatu-SP, 18618-000, Brazil

Recently, a significant progresse have been reached regarding the induction of multiple ovulations in mares. However, information about the hormonal implications of treatments is still needed. The goal of this experiment was to determine the serum concentration of progesterone (P_4) in mares, during transitional phase, submitted to a treatment with equine purified FSH (eFSH® - Bioniche Animal Health Inc., Canada) to induce superovulation. Twenty-six crossbred mares, from three to fifteen years old, were alternately assigned to on of two groups (n=13/group).At the moment the first follicle with a diameter equal or superior to 25mm was acquired, mares were assigned to group 1 (control) or group 2 (treatment with inter-muscular administration of 12,5 mg of eFSH, two times a day, until at lest half of the follicles larger than 30 mm had reached 35 mm in diameter). The induction of the ovulation(s) was performed with intravenous administration of 2500 IU hCG (Vetecor - CALIER do Brazil Ltda, São Paulo). Seven to eight days after the ovulation(s), blood samples were collected by jugular puncture. The samples were centrifuged and the serum stored at –16º C until laboratorial analysis performed by radioimmunoassay (Progesterone Kit Coat-A-Count – Diagnostic Products CO Medlab, São Paulo, SP, Brazil). Pearson's correlation coefficient was used to compare number of ovulation(s) and serum concentration of progesterone. Mares of the control group had just one ovulation and presented progesterone concentration equal to 7.39 ± 2.11 ng/ml. One mare from the treatment group had also just one ovulation and presented a similar concentration of progesterone (8.02 ng/ml) comparing to the group control However, the others mares of the treatment group had more than one ovulation, and progesterone concentration showed a positive correlation with the ovulation number ($p<0.001$), as follows: two mares had two ovulations and presented a concentration of P_4 equal to 14.53 ± 2,46 ng/ml; three mares had four ovulations and a P_4 concentration equal to 18.44 ± 2.57 ng/ml; three mares, had five ovulations with P_4 concentration equal to 24.38 ± 11.03 ng/ml and one mare had six ovulations and concentration of P_4 equal to 44 ng/ml. Moreover, three other mares had 10, 12 and 17 ovulations and P_4 concentrations of 24.9, 38.5 and 45 ng/ml, respectively. The detection of high concentrations of progesterone in mares submitted to eFSH® treatment witch presented multiple ovulations demonstrate that corpora lutea are functional. The high levels of progesterone observed in treated animal may be one of the reasons for the low embryonic recovery rates in superovulated animals due to a possible disturbed transport of oocytes and/or embryos in the oviduct.

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EFFECT OF DESORELIN AND EQUINE PITUTARY EXTRACT ON INDUCTION OF OVULATION IN CYCLING MARES

Melo, C.M.; Papa, F.O.; Medeiros, A.S.L.; Dell'Aqua Jr., J.A.; Carmo, M.T.; Araújo, G.H.M.; Alvarenga, M.A

Department of animal Reproduction and Veterinary Radiology - UNESP Botucatu SP Brazil.
malvarenga@fmvz.unesp.br

Timing of ovulation is a highly desirable event that has many advantages for the equine practitioner. To optimize the use of equine cooled or frozen semen and embryo transfer technique is necessary to induce ovulation to improve life span of spermatozoa. When breeding mares with cooled and frozen semen, it is imperative to use an ovulation-inducing agent in order to maximize the use of semen by minimizing the number of insemination doses per cycle. This would hopefully result in a reduction of the interval between insemination and ovulation. However, repeated injections induce antibodies against hCG, which is not desirable for induction of ovulation. Alternative ovulation inducing agents are useful for embryo transfer programs, especially for embryo donors, because these agents can avoid the induction of antibodies. Both GnRH and equine pituitary extract (EPE) are effective in inducing ovulation in a determinated period, without induce immunological resistance, thus they can be used during consecutives cycles. The aim of the present study was to verify the efficiency of GnRH and EPE at low concentration, on induction of ovulation. Seventy-nine cycling mares, at estrus with 35mm of diameter and uterine edema were used and received 1mg of desorelin intramuscularly or 10mg of EPE intravenously. After 24 hours of the induction of ovulation the mares were evaluated by ultrasound and then monitorated each 6 hours until ovulation. The results were aggregated in 5 groups according to the period comprehended between the administration until the ovulation: C1 (24 hr), C2 (between 24-36 hr), C3 (36-48 hr), C4 (48-60 hr) and C5 (more than 60 hr). The percentages for each group when desorelin was administrated were: C1 = 13.8%, C2 = 10.3%, C3 = 69.0%, C4 = 0% e C5 = 6.9% and for EPE: C1 = 17.5%, C2 = 45.0%, C3 = 35.0%, C4 = 2.5% e C5 = 0%. From those mares, 6.9% (2/29) were non responsive to 1mg of deslorelin. The mean interval to ovulation using 1mg deslorelin or using 10mg of EPE were respectively: 38.89 ± 7.38^{a} h and 34.75 ± 6.72^{b} h ($p<0.05$). These results suggest that both agents were efficient on the induction of ovulation. The EPE induces the ovulation 4 hours earlier than desorelin, and 10 mg of EPE is efficient to induce ovulation.

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HORMONAL LEVELS IN MARES SUPEROVULATED WITH PURIFIED EQUINE FSH – PARTIAL RESULTS

Machado, M.S.[1]; Araújo, G.H.M.[1]; Roser, J.F.[2]; Alonso, M.A.; Alvarenga, M.A.[1]

[1]Departamento de Reprodução Animal e Radiologia Veterinária - UNESP-Botucatu/SP, 18618-000-Brasil - [2]Animal Science, Endocrinology Laboratory, California University, Davis, USA.
misima@bol.com.br

The equine pituitary extract (EPE) is the only compound that consistently ovulations in mares. However despite of a good superovulatory response, the embryo recovery rate is low than expected. Studies performed in our laboratory have shown a better superovulatory response using purified equine FSH (Laboratório Bioniche-Canadá). The aim of this study is to evaluate the circulation hormonal levels of mares treated with purified equine FSH (Laboratório Bioniche-Canadá) compared with a non treated group, and with mares treated with Equine Pituitary Extract (EPE). Six mares in good nutritional and reproductive status were used. The same mares were used in all groups. GI (Purified equine FSH 12.5mg/IM, twice daily) and GII (control). These mares were monitored daily by ultrasound to detect ovulation, the treatment started on the 7th day after ovulation until the majority of follicles reached ≥35mm, at this moment, 3000UI hCG was administered. The blood samples to measure estradiol, inhibin and progesterone were daily collected since two days prior the begin of treatment until two days after the end of treatment. Hormonal evaluation was be performed at the Endocrinology Laboratory, of California University, in Davis. The profile of plasmatic concentrations of estradiol through treatment was similar to control, however the treated group concentrations were statistically higher than control on days -4, 0, 1 and 2 (day0=ovulation). Inhibin concentrations on group I (eFSH) presented a significant increase ($P<0.05$) beginning on day -7 till day 2, compared to control and the day of ovulation, it was observed a peak in the hormone dosages. Progesterone plasmatic concentrations do not have statistical difference among groups evaluated through the treatment period and ovulation, remaining low (lower than 1ng/ml) in both groups. The increase in estradiol concentrations observed in the superovulated group corresponds to an increase in number of pre-ovulatory follicles and their steroidogenic competence, as shown in cattle (Booth et al.1975). The significative increase in inhibin concentrations on treated group compared to control, observed beginning on day -7 could be related to the rise in follicle population during the follicular wave. Its peak observed on ovulation day is related to number of ovulations, because this hormone increases during ovulation, possibly due to extravagating follicular fluid to abdominal cavity (Ginther, 1992). These two hormone concentrations were similar during the oestrus cycle and could be related to number of follicles. These partial results, found in this study, allow us to conclude that endocrine estradiol and inhibin profiles in superovulated mares differ from control group proportionally to follicle numbers, showing that these follicles are capable of secreting inhibin and estradiol. This study is in progress to evaluate the profiles of proteic hormones (FSH and LH) and hormonal profiles from the same mares treated with EPE.

USE OF EQUINE PITUITARY EXTRACT AS AN AGENT TO INDUCTION OF OVULATION IN MARES

Medeiros, A.S.L.; Silva Jr., B.L.; Melo, C.M.; Carmo, M.T.; Papa, F.O.; Puoli Filho, J.N.P.; Araújo, G.H.M.; Alvarenga, M.A.

Department of Animal Reproduction and Veterinary Radiology – FMVZ/UNESP, Botucatu- SP, 18618-000, Brazil. malvarenga@fmvz.unesp.br

The use of drugs to induce ovulation plays a fundamental role on mare reproductive management, optimizing the results obtained from artificial insemination (AI) and from embryo transfer programs using either cooled or frozen- thawed semen. Human chorionic gonadotropin (hCG) is currently one of the most commonly hormone used in equine reproduction management showing numerous advantages with the majority of ovulations occurring within 48h from its administration. However, the induction of antibody formation is an inconvenient aspect of its use, this fact reduces efficiency after successive injections (Barbacini , et al.2000, Equine Vet Edu, 2:404-410). French researchers investigate the use of equine pituitary extract as an ovulation agent inductor in mares and concluded that the 25 or 50 mg dosage was efficient to induce ovulation (Duchamps et al., 1987. J. Reprod. Fert. Suppl., v.35. p.221-8). The present work aimed to compare low dosages of equine pituitary extract (EPE) to induce ovulation in mares during the breeding seasons of 2004 and 2005. Twenty-five mares were used, with a total of 97 cycles from which 40 cycles had ovulation induced with EPE 10mg (T1) and 57 cycles with EPE 5mg (T2) I.V. After estrus was verified, the mares were monitored once a day by rectal palpation and ultrasonography until a 35 mm follicle was detected. Ovulation was then induced using either T1 or T2, and the mares were monitored every 12 hours until the ovulation. The Ovulations occurred within the following observed periods: 12 to 24hs (P1), 24 to 36hs (P2), 36 to 48hs (P3) and 48 to 60hs (P4). The interval to ovulation differs between treatments within periods ($p<0,05$). At P1, 17,5%^{a} (7/40) of ovulation in T1 and 3.5%^{b} (2/57) of the T2, in the P2, 45%^{a} (18/40) of ovulations was detected of treatment T1 in comparison with 29.8%^{b} of T2, at P3, 66.7%^{a}(38/57) ovulations of T2 in comparison of 35%^{b} (14/40) in the T1, in the P4 only the T1 has a ovulation rate of 2.5% (1/40)**.** The mean of the time of detection of the ovulation were for the T1 and T2: 34.75 ± 6.72^{a} and 37.54 ± 3.05 hours ($p<0.05$). According with these results, both EPE doses were efficient to induce mare's ovulation the majority of ovulations within a period of 48, becoming an alternative as an agent to induce ovulation in programs requiring a precise interval, as using frozen semen in AI program. In most mares EPE was administered four consecutive cycles during the same breeding season and there was no reduction in its efficiency, this observation is certainly related with the fact that the drug is a homologous preparation.

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EFFECT OF LOW DOSES OF EQUINE PITUITARY EXTRACT AND EQUINE PURIFIED FSH ON OVULATION RATE AND EMBRYO RECOVERY RATE IN MARES

Rocha Filho, A.N.[1]; Lopes, E.P.[2]; Araujo, G.H.M.[1]; Alvarenga, M.A.[1]

[1]Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. [2]Haras Santa Esmeralda, Esmeraldas-MG, Brasil. arochavet@hotmail.com

The aim of the present study was to evaluate the efficiency of low doses of EPE and eFSH (Bioniche, Canada) to increase the ovulation and embryo recovery rates in mares. Mangalarga Marchador embryo donors were used from October to December. After an embryo collection on day 8 post-ovulation, the mares received an injection of dinoprost (7.5 mg/IM; Lutalyse, Pharmacia, Brasil) and were allocated in one of two experimental groups: EPE (n=11) or eFSH (n=14). For all mares two intervals between one embryo collection and the subsequent ovulation were studied, so that the first one was the control cycle (untreated) and the second was the treated cycle. In both cycles per rectum ultrasonography of the ovaries and uterus was performed daily. During estrus the mares were bred every 48 h with fresh semen of the same stallion and received hCG (2,500 IU/IV; Vetecor, Lab. Calier, Brasil) upon detection of a 35 to 40 mm follicle. Treatments consisted of EPE (4 mg/IM/s.i.d.; Grupo EPE) or eFSH (5 mg/IM/s.i.d.; Grupo eFSH) administration from day 8 (day 0 = ovulation) until the day before the ovulations were induced with hCG. Embryo collections were performed on day 8 post-ovulation and always followed by administration of luteolytic. The percentage of mares with more than one ovulation was compared by means of Fischer's test and the mean number of ovulations and embryos by paired Student *t* test between cycles within the same group and by unpaired Student *t* test between different groups, at a 5% error probability. No significant difference was observed for the percentage of mares with more than one ovulation between EPE and eFSH treatments (36.4% and 50.0%, respectively). Higher ($P<0.05$) mean number of ovulations and recovered embryos was observed in the EPE-treated cycle compared to its control (1.36 ± 0.5 *vs.* 1.0 ± 0.0 and 1.18 ± 0.6 *vs.* 0.63 ± 0.5, respectively). The eFSH treatment resulted in higher ($P<0.05$) mean of ovulations compared to the control (1.64 ± 0.7 *vs.* 1.0 ± 0), with no significant difference in the mean number of embryos recovered (1.0 ± 0.7 and 0.78 ± 0.4, respectively). Embryo recovery rates in relation to the number of ovulations differed ($P<0.05$) between the EPE and eFSH treatments (87.5% e 60.8%, respectively). However, there was no significant difference between EPE and eFSH treatments regarding the percentage of embryos recovered per flush (127.3% and 100%, respectively). In conclusion, administration of low doses of EPE or eFSH to mares increases the mean number of ovulations in the herd and allows for the recovery of one embryo per flush on average. It is necessary to improve the protocols in order to guarantee that a higher percentage of individuals will respond to treatment.

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EFFECT OF OVULATION INDUTION WITH HUMAN CHORIONIC GANADOTROPIN ON PROGESTERONE PLASMATIC LEVELS AT EARLY DIESTRUS IN MARES

Araujo, G.H.M.[1]; Dutra, F.O.[2]; Carmo, M.T.[1]; Oba, E.[1]; Alvarenga, M.A.[1]

[1]Department of Animal Reproduction and Veterinary Radiology – FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. [2]Haras Estrela do Oriente - Três Rios-RJ, Brazil. arochavet@hotmail.com

The present experiment aimed to evaluate the interrelation between the ovulation induction with hCG and the raise in the plasmatic levels of progesterone and to investigate the possibility that mares with induced ovulations could be used earlier than other mares as embryo recipients. The present study was performed during the reproductive season of 2003-2004 and 42 Mangalarga Marchador mares were used. All mares were healthy and reproductively active and were divided in two groups: Control (n=22), with non-induced ovulations; and hCG (n=20), using hCG as an ovulation inductor (2500 UI, IV, Vetecor, Lab. Calier-Brazil) upon detection of a follicle with 35 to 40 mm in diameter. The mares of both groups were daily evaluated by rectal ultrasonography until detection of ovulation. After that, daily blood samples were collected until day 3 post-ovulation. The blood samples were centrifuged and the plasma frozen until plasmatic progesterone was determined by means of RIA. The comparison of the plasmatic progesterone among different days within the same group was performed by ANOVA and Tukey and to determine daily differences between groups the Student *t* test was used, both at a 5% error probability. Statistical difference was observed for the progesterone concentrations among the analyzed days (D0, D1, D2 and D3) within the same group. Mean daily concentraions of progesterone (ng/ml) in the control and hCG groups were: D0 0.33 ± 0.055 and 0.594 ± 0.162; D1 1.001 ± 012 and 1.302 ± 0.246; D2 2.348 ± 0.243 and 2.607 ± 0.236; D3 4.115 ± 0.431 and 4.35 ± 0.433, showing no statistical differences between the groups in each day analysis. Percent differences were found in the mean daily values of progesterone between groups: 80% on the ovulation day (D0), 30% on D1, 11% on D2 and 5.7% on D3, showing higher numerical progesterone levels on the day ovulation was detected. It was concluded that there is not a sufficient increment on the plasmatic levels of progesterone in mares treated with hCG to permit their use as embryo recipients earlier than control mares. The maintenance of the numeric higher levels sooner after ovulation may be due to the half life of hCG, that probably does not last long after ovulation. This observation is currently under study.

EARLY EMBRYONIC DEATH IN ANOVULATORY RECIPIENTS MARES SUPPLEMENTED WITH LONG ACTING PROGESTERONE

Testa, A.C.[1]; Carmo, M.T.[2]; Alvarenga, M.A.[2]

[1] Equine Practitioner, Nova Friburgo, RJ, Brazil [2]Department of Animal Reproduction and Veterinary Radiology, Faculty of Veterinary, FMVZ – UNESP – Botucatu, SP, Brazil.
testavet2004@yahoo.com.br

Utilization of anovulatory mares treated with progestagens as embryo recipients is desirable in embryo transfer programs, especially at the beginning and the end of the breeding season when a smaller number of recipient are cycling when compared with the donor mares. The utilization of noncycling progesterone-treated recipient mares has become a routinelly procedure in Brazil. However there is few informations about the early embryonic death rates (EED) in noncycling progesterone-treated recipient mares. The present study aimed to evaluated the incidence of EED in mares recipient mares supplemented with long acting P4. A total of 34 recipient mares (20 cycling and 14 non cycling) were used. The anovulatory mares were examined at regular intervals and characterized by absence of a corpus luteum and presence of follicles <20 mm in diameter in subsequent examinations. After 3 days of estradiol cypionate (ECP) administrated in decreasing doses (5 mg, 3 mg and 2mg /day im). Only mares with good uterine edema after the third day of ECP treatment were used. Anovulatory recipients were supplemented with 1500mg of long acting P4 (BET Lab,USA) for 4 to 6 days before embryo transfer . Day 8 embryos were transferred non-surgically into recipients 4 to 6 days after the beggining of progesterone supplementation. Non cycling mares were treated with 1500 mg long acting progesterone every 7 days untill 120 days of pregnancy. Pregnancies were checked on days 12 and 50, after doonor ovulation, by transrectal ultrasonography. Data were analyzed by Chi-square. The incidence of early embryonic death between 12 and 50 days were similar (P>0.05) for control mares (15%; 3/20)and P4 treated mares (13%; 2/14). We concluded that the supplementation at each 7 days with long acting progesterone is a safe procedure and was able to maintain pregnancy in noncycling recipients mares.

COMPARISION BETWEEN TWO DIFFERENT MEDIA FOR EQUINE EMBRYO TRANSFER IN MANGALARGA MARCHADOR MARES.

Caiado, J.R.C.[1]; Fonseca, F.A.[1]; Silva, J.F.S.[1]; Fontes, R.S.[1]

[1]Norte Fluminense State University – Agriculture Science and Technology Center. Avenida Alberto Lamego-100. Campos dos Goytacazes-RJ. jrccaiado@hotmail.com

Different buffers are used in the media for washing and embryo maintenance during the procedures of equine embryo transfer. The buffers carbonate, phosphate, and zwitterionic are the most used. During the procedures of transference, it is common to keep the embryos into the maintenance media during a time longer than previewed. This experiment was realized with the objective of comparing the costs and benefits of using two media with different buffers for embryo transfer in Mangalarga Marchador breeding mares. The medias are DPBS (phosphate) and Embriocare (zwitterionic). The embryos were divided randomly in two groups, A (zwitterionic) and B (phosphate), and kept in the media for different periods of time (0, 30, 60, and 120 minutes) in temperature between 28 and 35°C. In the group A (zwitterionic), the distribution was as following: 15 embryos were kept during 0 minute, 15 embryos during 30 minutes, 16 embryos during 60 minutes, and 14 embryos during 120 minutes. In the group B (phosphate), 13 embryos were kept during 0 minute, 18 embryos during 30 minutes, 16 embryos during 60 minutes, and 19 embryos during 120 minutes. The results indicate that both groups A and B are equally efficient ($P>0.05$), for qui-square method, in the processes of scanning, washing, maintenance at ambient temperature for different time periods (0 to 120 minutes), and inovulation of equine embryos under tropical conditions. The pregnancy rates using the media zwitterionic and phosphate were 65% and 69.7% in the different periods. Since Embriocare is more expensive, DPBS presented the best cost-benefit ratio.

USE OF hCG AIMING AT IMPROVEMENTS OF SOME REPRODUCTIVE CHARACTERISTICS OF EMBRYOS RECIPIENTS MARES

Sousa, F.A.C.[1]; Fleury, P.D.C.[2]; Alonso, M.A.[2]; Arruda, R.P.[1]

[1] Centro de Biotecnologia em Reprodução Animal - VRA/FMVZ/USP, Pirassununga-SP, 13630-000, Brasil. [2] Central Fleury de Reprodução Eqüina, São José do Rio Pardo-SP, Brasil.
dr.fer@terra.com.br

The objective of this study was evaluated the impact of hCG on reproductive characteristics of embryos recipients mares. 84 Mangalarga breed recipients had been used, between 4 and 12 years, share aleatoric in 3 groups, with 28 animals each, that had received the following treatments: (G1) 1ml of physiological solution, IM, when dominant follicles =35mm; (G2) 2500UI of hCG (Vetecor®, Calier, Barcelona, Spain), IM, when dominant follicles =35mm and (G3) 2500UI of hCG, IM, one day after the ovulation (D1). Blood samples had been collected in the D0, D2, D4, D6, D8, for dosages of progesterone. In D6 all the recipients had been evaluated for this reproductive characteristics: tone uterine and cervical, evaluated by palpation per rectum and receiving scores of 1 (minimum) to 4 (maximum), uterine morphoechogenicity, visualized by ultrasound, classified in 1 (minimum/homogeneous image) to 4 (maximum/heterogeneous image) and of the corpus luteum, classified in corpus luteum whith echogenical center or not, and order of choice of the recipients, that varied of 1º (better) to 4º (worse), classified subjectively based in the evaluations above cited. It was evaluated by ANOVA and the test of qui-square, contained in SAS program. Statistical differences had been found (P=0.05) in the plasmatic concentrations of P4 in the D2 between the G1 and G2, (3.8ng/mL and 5.65ng/mL, respectively) and in the D4, where all groups had differed between itself (G1:8.42ng/mL, G2:16.01ng/mL and G3:14.85ng/mL). Statistics differences was detected enter the groups for uterine tone (P=0.05), the G3 presented low occurrence of tone 1 and high of tone 4. In relation to cervical tone statistics differences existed (P=0.05), Occurring high frequency of tone 1 in the G1, of tone 2 in G2 and tone 4 in G3 and low frequency of tone 2 in G3. About the uterine morphoechogenicity, the G1 presented high occurrence of 3 and low of 2; In G3 occurred low of 3, presenting statistical differences (P=0.05) between them. Statistical differences in the morphoechogenicity of the corpus luteum between the groups had not been observed (P>0.05). It had differenced in the order of choice of the recipients between the groups (P=0.05), was observed low number of mares of 1º in the G1 and of 4º choice in the G3; high number of mares of 4º choice in the G1, 2º choice in the G2 and 1º choice in the G3. Concludes that the treatment with hCG in embryos recipients with follicles =35mm, or in the D1, improves the reproductive characteristics, therefore availability of a bigger number of females apt to receiving embryo.

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ENDOMETRIAL BIOPSY SCORE AND REPRODUCTIVE APTITUDE IN EMBRYO RECIPIENT MARES. PRELIMINARY RESULTS

Losinno, L.; Alonso, C.; Castaneira, C.

Cátedra de Producción Equina, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, (5800) Río Cuarto, Argentina. llosinno@ayv.unrc.edu.ar

In the last decades reproductive efficiency in equines was improved with embryo transfer and other biotechnologies, most of them using recipient mares for embryos of valuable donors. Actually, recipient mare is one of the most important factor in the success of commercial equine embryo transfer program. This study aims to correlate the endometrial score with age in mares selected for recipients. The objective was to test the hypothesis that routine reproductive clinical examination in the mare is not a better predictor of reproductive aptitude in the selection of recipients for embryo transfer programs. Seventy five endometrial biopsies of selected recipient mares from commercial equine embryo transfer centers of Argentina were analyzed. All mares were submitted to a standard clinical examination of reproductive status by veterinarians, including: age, body score, no signs of systemic disease or lameness, rectal palpation and ultrasound of reproductive tract. Two veterinarians from Equine Reproduction Laboratory, double blind classified the endometrial biopsy samples according Kenney and Doig (1986). Endometrial scores were compared between age groups using CATMOD (SAS) procedure. The results were expressed in groups of age and corresponding endometrial scores (%): 2–4 years (n=9), I(55.5), IIa(22.2), IIb(11.1), III(11.1); 5–7 years (n=14), I(35.7), IIa(50), IIb(14.3), III(0); 8–10 years (n=32), I(31.2), IIa(25), IIb(37.5), III(6.2); 11–13 years(n=4), I(0), IIa(50), IIb(25), III(25); 14–16 years(n=10), I(10), IIa(20), IIb(50), III(20); 17–19 years (n=6), I(0), IIa(16,6), IIb(50), III(33.3). When the results were expressed in two groups of age: Group A: < 14 years (n=59) and Group B: ≥ 14 years (n=16) the biopsy scores for both groups respectively (%) were: I (33.9 and 6.5), IIa (33.9 and 18.5), IIb (27.1 and 50), III (5.1 and 25). The significant differences between endometrial scores and ages suggest that in coincidence with previous reports, endometrial quality were negatively correlated with age. Considering the degenerative changes in endometrium and its relation with less reproductive performance, mares of IIb and III score should be excluded as recipients in a embryo transfer program, independently of its age and clinical score. In the “young” mares (group A), score IIb and III were significantly smaller than in old mares (32.2 vs. 75 %; $p<0.05$) but this percentage will be very important in terms of negative economic impact in embryo transfer programs. In this study, 32% of the mares of age less than 10 years that were approved by gynecological and clinical standard examination, will be seriously compromised in its ability of develop a pregnancy to term. Considering this preliminary results we conclude that mares ≥14 years of age should be excluded as recipients of embryos, and mares < 14 years should be passed a complete reproductive exam, including endometrial biopsy.

EFFECT OF COOLED SEMEN ON EQUINE EMBRYO RECOVERY RATE

Duarte, M.B.[1]; Vieira, R.C.[2]

[1]Médico Veterinário, Mestre, Pça das Rosas 80, Uberaba; Brasil.
[2]Departamento de Produção Animal, UFU, Uberlândia; Brasil. mb.duarte@terra.com.br

The use of cooled semen is another choice to enhance the options for coupling in equine embryo transfer. The objective of this study was to evaluate the effect of cooled semen on equine embryo recovery rate. The study was conducted on 187 Quarter Horse and Paint Horse donors with ages from two to 25 years old, during the breeding season of 2001/2002 to 2004/2005. The ovaries were monitored by ultrasonography after the beginning of the heat and when the follicle measuring =35 mm and moderate uterine edema was present the mares were distributed into two groups. In Group I the mares were inseminated every 48 hours up to ovulation using fresh semen with an inseminating dose of approximately 1.0 x 10^9 progressive spermatozoa. In Group II they were treated with one dose of 2000 ui hCG IV and inseminated once, 24 hours after with cooled semen with minimum inseminating dose of 1 x 10^9 spermatozoa. The semen was diluted in skim milk extender (Kenney) and kept at 18°C in an appropriate box (Max Semen*). The insemination was made between 12 to 24 hours after collection. The embryos were collected 8 days after ovulation, in expanded blastocyst stage, with Ringer lactate solution. A total of 971 uterine lavages were made, being 886 in Group I and 85 in Group II. 671 embryos were recovered, being 626 (70,6%) in Group I and 45 (52,9%) in Group II. The results were analyzed statistically by the chi-square test. The embryo recovery rates using fresh semen was higher ($P<0.05$) when compared to cooled semen (70.6% and 52.9% into Groups I and II, respectively).

*EHG Agrofarma, SP, Brazil.

Key word: Equine, cooled semen, embryo recovery.

EFFECT OF DONOR AGE ON EQUINE EMBRYO RECOVERY RATE

Duarte, M.B.[1]; Vieira, R.C.[2]

[1]Médico Veterinário, Mestre, Pça das Rosas 80, Uberaba; Brasil.
[2]Departamento de Produção Animal, UFU, Uberlândia; Brasil. mb.duarte@terra.com.br

The objective of this study was to evaluate the effect of donor age on equine embryo recovery rate. The study was conducted on 152 Quarter Horse and Paint Horse donors with varied ages were used during the 2001/2002 to 2004/2005 breeding season. The ovaries were monitored by ultrasonography after the beginning of the heat. When a follicle measuring =35 mm was present the mares were inseminated with fresh semen every 48 hours up to ovulation, with approximately 1.0 x 109 motile spermatozoa dose. According to ages, the mares were distributed into three different groups, Group I from two to four years of age, Group II from four to 16 years and Group III beyond 16 years. The embryos were collected 8 days after ovulation, in expanded blastocyst stage. Up to six uterine lavage with Ringer lactate solution were made, using successive 500 ml infusions in Group I and 1000 ml in Group II and III, until the embryo recovered. A total of 886 uterine lavage were made, being 85 in Group I, 653 in Group III and 148 in Group III. 626 (70.6%) embryos were recovered, being 69 in Group I (81.1%), 478 (73.2%) in Group II and 79 (53.3%) in Group III. The results were analyzed statistically by the chi-square test. The embryo recovery rate in Group I and II was higher ($P<0.05$) when compared to the animals in Group III (81.1% and 73.2% versus 53.3% into Groups I, II and III respectively).

Key word: Equine, age, embryo recovery.

EFFECT OF DONOR MARE'S AGE IN EMBRYONIC LOSS RATE

Alonso, M.A.[1]; Fleury, P.D.C.[1]; Neves Neto, J.R.[1]; Machado, M.S.[2]

[1] Fleury Reprodução Equina – São Jose do Rio Pardo- SP, 13720-000, Brasil.[2] Departamento de Reprodução Animal e Radiologia Veterinária - UNESP-Botucatu/SP, 18618-000-Brasil.
gutalonso@gmail.com

Aged mares represent a great number of the donors in an embryo transfer program, mainly because these animals have a high quality progeny or have had good performance in sports. Data on pregnacy loss rates, from aged maré embryos are conflicting. For this reason, this study aimed to evaluate embryo loss rate comparing aged and Young donors. Data collected from three different breeding seasons were used, 2002/2003, 2003/2004, 2004/2005, in a commercial embryo transfer program at Fleury Reprodução Equina. The donors from whom embryos were collected were of different breeds as Mangalarga Paulista, Mangalarga Marchador, Árabian, Quarter Horse, pólo Thoroughbred and Campolina. The ages were between 2 to 26 years old. Embryos were recovered nonsurgically 6 to 9 days after ovulation. Upon identification, embryos were assessed for size, grade and developmental stage. Embryos were either transferred immediately or maintained at 15 a 18º C from 2 to 24 hours. Embryos were transferred nonsurgically. Recipients were Mangalarga Paulista or cross-bred, between3 to 14 years of age, barren or wet. Recipients were used 3 to 9 days post ovulation and selected by uterine tone and echogenicity. Pregnancy was first detected by ultrassonography on days 13 to 16 after the donor's ovulation and it was confirmed on day 40 of pregnancy. The embryos used were 453. Donor mares were separated in two different groups according to age: Group I, mares 2 to 17 years old and Group II, mares 18 or more years old. In GI, there were 408 embryos, while in GII there were 45 embryos from 27 different donors. In order to compare pregnancy rates among groups, the Chi-Square test was used. Results obtained on pregnancy loss were in GI , 12.5% and in GII, 13.2%. No statistical difference ($p>0.05$) among groups was detected and values were comparable to those observed in literature in commercial embryo transfer programs. Even though the recipient, the ovulatiob\n synchrony, age or diameter of embryo haven't been considered, the results obtained indicate that recipient mares that received embryos from aged or young mares have the same pproblability of having an embryo loss till 40 days of gestation.

PREGNANCY RATES AFTER NONSURGICAL TRANSFER OF EQUINE EMBRYOS STORED IN EMCARE OR VIGRO HOLDING MEDIA FOR 18H AT 15 TO 20ºC

Fleury, P.D.C.[1]; Neves Neto, J.R.[1]; Alonso, M.A.[1]; Squires, E.L.[2]

[1] Fleury Reprodução Equina – Sao Jose do Rio Pardo- SP, 13720-000, Brasil.[2] Animal Reproduction and Biotechnology Laboratory Colorado State University, Fort Collins, CO 80523, USA.
fleury@rantac.com.br

Embryos can be collected and shipped at 5°C or 15 to 20°C to recipients within 24 hr for transfer. Holding media for embryos have been Ham's F10, Vigro or EmCare. Limited studies have compared viability of embryos after storage in various holding media for greater than 12 hr. The objective of this study was to compare pregnancy rates after nonsurgical transfer of embryos that had been held in either Vigro or EmCare holding media for 18 hr at 15 to 20°C. Donor mares were of different breeds, age between 3 to 26 years old, maintained in different breeding farms and in Fleury Reprodução Equina Center. Embryos were recovered nonsurgically 7 or 8 days after ovulation. Upon identification, embryos were assessed for size, grade and developmental stage. Only grade I embryos were used in this experiment. After that, they were "washed" by transferring it sequentially through at least ten 1ml drops of either Vigro (Bioniche Animal Health, Athens, GA) or EmCare (Professional Embryo Transfer Supply, Irving, TX) holding media in a Petri dish .The embryos were loaded inside 5-ml tubes containing 4 ml of the same media used for "washing". The plastic cooler had a ice pack wrapped with newspaper. Newspapers were layered on top the ice pack and the embryo tube, wrapped in 2 sheets of paper, was placed on top. Embryos were then stored for 18 hr prior to non surgical transfer into synchronized recipients, from day 3 to 9 days after ovulation. Recipients were from different breeds (age 3 to 14 years old). All of them were at Fleury Reproduction Equine Center. Ultrasound was used to detect pregnancy on days 14 to 16 after donor's ovulation. Data was analyzed by Chi-square test. Pregnancy rates were similar for embryos stored in EmCare (15/22, 68%) or in Vigro media (18/21, 86%). In conclusion, both holding media were effective in maintaining the viability of embryos for 18 h prior to transfer. Although fresh embryo transfers were not included, these pregnancy rates are similar to those reported after fresh equine embryo transfer.

FIRST BIRTH OF TWO FOALS FROM HORSE EMBRYO SPLITTING IN LATIN AMERICA

Silveira, L.L.[1,2]; Pimentel, C.M.[1]; Melo, N.S.S.[1]; Souza, R.V.[2]; Rumpf, R.[2]

[1] Universidade de Brasília-UnB/Faculdade de Agronomia e Medicina Veterinária-FAV, 70910-900 Brasília-DF, Brasil; [2] Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, 70770-900 Brasília-DF, Brasil. silveiraleoluiz@yahoo.com.br

The first two foals were born in Latin America on the Sucupira Experimental Station after splitting horse embryos of Embrapa-Genetic Resources and Biotechnology, Brasilia-DF. They are called White and Snow due to their coat colour and with reference to Snow White and the Seven Dwarfs. White was born on the 23rd of December 2004 and Snow on the 4th of January 2005, weighing 52 and 54 kilos respectively. The identical twins were generated in different uteri, from a single embryo, which was divided in two equal parts and transferred into the receptor mares. Embryo splitting presents a victory for the country, as much from a commercial point of view as from a conservation point, as it allows for the increase in number of descendents from a high quality animal more quickly and efficiently. This is an especially important technique for horses, as it can be used along with super-ovulation and frozen embryos. Embryo splitting may become a viable alternative to increase the number of foals per donor/year. Identical twins can be used in research in the areas of immunology and pharmacology, as well as in studies to evaluate the influence of the environment on animal performance.

GYNECOLOGYCAL STUDY OF FEMALE BOVINES DESTINATED TO ARTIFICIAL INSEMINATION PROGRAMS OR AS EMBRYO RECEPTORS: PRELIMINARY RESULTS

Del Rei, A.J.[1]; Bartolomeu, C.C.[2]; Neves, A.L.A.[1]; Balbino, S.C.[1]; Ferraz, P.C.[1]

[1]Centro Biotecnológico de Reprodução Animal, CBRA, DTRA, UESB - Itapetinga-BA, 45 700-000, Brasil,[2]DCAA, CMV-UESC delrei@uesb.br

With the objective of determine the prevalence of the various morpho-phyisiological ovarian presentation in bovine females to establish a criterion of selection by the ovarian status for the employment in AI programs or as embryo transfer receptors there were examined 10,578 females of different genetic groups (beef, milk and their crosses) raised in different regions of the south and southeast of the Bahia state in the period of 2001 to 2004. The exams were done by rectal palpation. The ovaries were classified by size, consistence, morphology and aspect, being stratified into 16 combinations. The results were presented by frequency and percentage into the established categories for this study. From the total of females evaluated, 6,040/10,578 (57.1%) were pregnant and 4,538/10,578 (42.9%) were not pregnant. From the non pregnant group there were randomly sampled 1,101 (24%) without apparent anomalies in the uterus and ovaries, totalizing 2,202 ovaries evaluated. The ovaries were classified as voluminous (v), small (sm), soft (sf), hard (h), rounded (r), flatted (f), smooth (sh), rough (rh). From the results, there were established the possible combinations: v, sf, r and rg (571 – 25.9%); v, sf, r, and sh (295 – 13.4%); v, sf, f, and sh (230 – 10.4%); v, h, r and sh (210 – 9.5%); v, h, f and sh (159 – 7.2%); v, h, f and rh (120 – 5.4%); v, sf, f and rh (96 – 4.4%); v, h, r and rh (53 – 2.4%); sm, sf, r and sh (90 – 4.1%); sm, h, r and sh (87 – 3.9%); sm, sf, f and sh (73 – 3.3%); sm, sf, r and rh (27 – 1.2%); sm, h, f and sh (38 – 1.7%); sm, h, f and rh (57 – 2.6%); sm, sf, f and rh (42 – 1.9%) and sm, h, r and rh (54 – 2.5%). Based on the findings, in a second moment, there will be established among the various combinations, which will present the best results in the AI program and as embryo receptors.

AVERAGE NUMBER AND SIZE OF OVARIAN STRUCTURES FROM NULLIPAROUS GIR AND HOLSTEIN EMBRYO DONNORS

Ferreira, M.B.D.[1]; Lopes, B.C.[2]; Souza, J.C.[3]; Azevedo, N.A.[4]; Ordones, F.[4]

[1] EPAMIG – Triângulo and Alto Paranaíba Technological Center CP.351, 38001970 – Uberaba, MG, brandao@epamiguberaba.com.br - [2] DSc., MSc., Professor of Animal Science – FAZU; biabrand@uai.com.br - [3] Ph.D. Professor of Animal Science at UFLA; jcamisao@ufla.br - [4] Embryo Transfer Specialized Veterinarian.

This study was conducted during the breeding season of 2003 at EPAMIG, Sete Lagoas MG. The size and average number of ovarian structures of Holstein and Gir heifers were evaluated. The analysis were performed on the first day of superovulation treatment, between days 8 and 12 of the estrous cycle, at the day of donnor estrus and on the day of flushing. Gir (33.63±4.46 months old) and Holstein (22.27±3.68 months old) heifers were synchronized with 500 µg of Cloprostenol, given intramuscularly (im) 11 days apart. Gir and Holstein heifers received, respectively, 100 and 200 IU of FSH (Pluset®), during four consecutive days, in 12 hour apart decreasing injections (im). Heifers were re-synchronized for the 2nd and 3th flushes. Ovarian length, width and height, as well as non-ovulatory follicles, follicles (≥ 3 < 7 mm and ≥ 7 mm) and corpora lutea (CL) were measured by ultrasonography. Means were compared by the T test. Right ovary diameters (cm) were 3.03±0.57 and 3.55±0.81 at the day of superovulation, 3.43±0.74 and 3.98±0.74 at the day of estrus, and 2.99±0.92 and 2.96±0.70 at the day of flushing, for gir and holstein heifers, respectively. Right ovary areas (cm^2) were 2.38±0.55 and 2.33±0.56 at the beginning of superovulation, 2.70±0.91 and 2.86±0.55 at the day of estrus, and 2.99±0.92 and 2.96±0.70 at the day of flushing for gir and holstein heifers, respectively. Left ovary diameters (cm) were 2.59±0.48 and 2.90±0.88 at for superovulation onset, 2.75±0.49 and 3.23±0.92 at the day of estrus and 3.90±1.24 e 3.73±1.42 at the day of flushing, for gir and holstein heifers, respectively. Left ovary areas (cm^2) were 1.94±0.31 and 2.03±0.47 at superovulation onset, 2.55±0.40 and 2.34±0.72 at estrus day and 2.81±0.85 and 2.43±0.81 at the day of flushing for gir and holstein heifers, respectively. CL diameters (cm) were 2.07±0.58 and 2.35±0.36; luteal cavity diameters (cm) were 0.58±0.36 and 0.83±0.40 and percentages of cavitary in the CL were 16.6 and 22.2 % for gir and holstein heifers, respectively. CL areas (cm^2) were 2.99±0.54 and 4.21±1.30 at the day of superovulation and number of palpable CL at flushing were 6.41±5.72 and 6.00±4.16 respectively for gir and holstein heifers. Mean follicle (= 3 < 7 mm) numbers were 14.07±5.80 and 9.60±4.46 at superovulation onset and 13.42±6.16 and 13.90±9.2 at the day of estrus for gir and holstein heifers, respectively. Mean follicle ((7 mm) numbers were 3.69±3.77 and 3.20±1.42 at the beginning of superovulation, 9.57±3.90 and 13.45±9.6 at the day of estrus, and non-ovulated follicle numbers were 2.25±2.59 and 2.90±2.51 for gir and holstein heifers, respectively. Mean number of flushed structures, combined for both breeds, was 6.21±4.32, of which 3.57±3.08 were viable embryos. With the exception of CL area, all other measurements did not differ between groups. It can be concluded that FSH stimulated heifer ovaries similarly and adequately in both breeds.

Key words: heifer, superovulation, embryos, follicles, corpus luteum.

OVULATORY CAPACITY IN *Bos indicus* HEIFERS

Gimenes, L.U.[1]; Carvalho, N.A.T.[1]; Sá Filho, M.F.[1]; Santiago, L.L.[1]; Carvalho, J.B.P.[2]; Mapletoft, R.J.[3]; Barros, C.M.[4]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ – USP, SP, 05508-000, Brasil; [2]Pólo Regional de Pindamonhangaba (APTA), SP, Brasil; [3]Western College of Veterinary Medicine, University of Saskatchewan, Canadá; [4]Departamento de Farmacologia, IBB – UNESP – Botucatu, SP, Brasil. ligimenes@usp.br and barusell@usp.br

In Holstein multiparous the ovulatory capacity is acquired when follicle reaches diameter approximately 10mm, about 1.5mm after follicular deviation (Sartori et al., Biol. Reprod., v.65, p.1403-09, 2001). The aim of present study was to evaluate the diameter in which dominant follicle of *Bos indicus* heifers acquire ovulatory capacity after LH challenge. Twenty-nine cycling cross-bred *Bos indicus* heifers were kept on pasture, weighing and aging above 350kg and 24 months, respectively. The animals were previously synchronized with a progestin plus estradiol benzoate protocol. After ovulations (Day 0), the follicles were monitored by ultrasonography every 24h, until diameters achieved were 7-8.4mm (n=9), 8.5-10mm (n=10) and >10mm (n=10). In order to verify ovulatory capacity, the animals were treated with 25mg of LH (Lutropin-V®, Bioniche, Canada). From the treatment, ovaries were scanned by ultrasonography each 12h during 48h. Statistical analysis was performed by Anova, Bartlett and Chi-square tests. The mean follicular diameter (mean±SEM) at LH administration was 0.76^{a}±0.01cm in 7-8.4 group; 0.96^{b}±0.01cm in 8.5-10 group and 1.09^{c}±0.02cm in >10 group ($P<0.05$). The ovulatory rate after LH challenge was: $33.3\%^{a}$ (3/9); $80.0\%^{b}$ (8/10) e $90.0\%^{b}$ (9/10; $P<0.05$) in 7-8.4; 8.5-10 and >10mm groups, respectively. The moment of ovulation was 38.0±4.0h; 31.5±2.7h and 30.0±2.0h ($P>0.05$) to the same groups, respectively. In conclusion, acquisition of ovulatory capacity in *Bos indicus* heifers increases after follicles achieve 8.5-10mm diameter, which is inferior to that observed in Holsteins (*Bos Taurus*).

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ULTRASONOGRAPHIC STUDY OF FOLLICULAR DEVIATION IN NELORE (*Bos indicus*) HEIFERS

Gimenes, L.U.[1]; Sá Filho, M.F.[1]; Madureira, E.H.[1]; Trinca, L.A.[2];
Barros, C.M.[2]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ – USP, SP, 05508-000; [2]Departamento de Farmacologia e Bioestatística , IBB – UNESP – Botucatu, SP. ligimenes@usp.br and barusell@usp.br

The ovarian follicles in cattle present a wave growth pattern, in which a cohort of follicles emerge and at determined time, one of them stands out continuing to grow and becomes the dominant follicle. In this study follicular diameter (∅) and moment (DEVM) related to follicle deviation on first-wave of Nelore heifers were evaluated. Thirteen cycling heifers, kept on pasture, weighing above 325 kg and aging between 20 to 24 months were used. On random day of estrous cycle, animals were treated with 2mg of estradiol benzoate (EB) i.m. (Estrogin®, Farmavet, Brasil) and an auricular Norgestomet implant (Crestar®, Intervet, Brasil). Eight days later, implants were removed and 0,15mg of d-cloprostenol i.m. (Preloban®, Intervet, Brasil) was injected. Twenty four hours after implant withdrawal, females received 1mg of EB i.m. From implant removal, the heifers were examined by transrectal ultrasonography (Aloka SSD-500, Japão) each 12h until sixth day of estrous cycle (Day 0 – Ovulation day) to verify ovulation moment, DEVM and ∅ at follicular deviation. Ovaries were mapped, registering the diameter of three largest ovarian follicles. Retrospectively two largest follicles were determined, being classified as dominant follicle (DF) and subordinate follicle (SF). DEVM was defined as the first examination in which DF growth was superior to SF (Ginther, Biol.Reprod., v.65, p.638, 2001). After find DEVM, the DF and SF ∅ were determined. Follicle deviation moment occurred 64,6±5,5 hours after ovulation and dominant and largest subordinate follicle diameters were 0,62±0,02 and 0,58±0,02 (cm), respectively.

EFFICIENCY OF ARTIFICIAL INSEMINATION IN FIXED TIME COMBINED WITH PROGESTERONE INTRAVAGINAL DEVICE AND FERTILITY RATES OF FEMALE BUFFALOES RAISED IN DIFFERENT PRODUCTION SYSTEMS.

Ribeiro, H.F.L.; Picanço, N.S.; Rolim Filho, S.T.; Pinho, R.O.; Araújo, C.V.; Vale, W.G.

Setor de Reprodução Animal, ISPA / UFRA. Belém-PA, Brasil. haroldo.ribeiro@ufra.edu.br

The buffalo extensive breeding system used in Amazon region is responsible for a prolonged postpartum anoestrus which play an important role in the reproductive efficiency of this species. With the aim of increase the pregnancy rate accomplished with the use of steroid protocolls and artificial insemination in fixed time (AIFT) in female buffaloes raised in semi-extensive (Experiment I) and extensive system (Experiment II), respectively. In the experiment I, 29 buffaloes were used and it was divided in two Groups: (G1) with 14 female buffaloes which in the day 0 received a PRID with 1,0 g of Progesterone (Cronipres®) and 2ml (IM-intra-muscular) of Estradiol Benzoate (EB), (Estrogin®); after 9days of the PRID withdraw it was injected through vulvar intra-submucosa a reduced dose of 1ml/7,5mg of PGF2α (Croniben®) followed by a second dose injection IM 1,0 ml of (EB). An AIFT was performed 24 h after EB injection. The (G2) with 15 female buffaloes received the same treatment of G1 with exception of the PRID. In the Experiment II, (G2) with 29 female buffaloes which in the day 0 received a PRID with 1,0 g of Progesterone followed by an injection of 1ml/100μg(IM) of gonadorelin.After 7 days thePRID was withdraw followed by an injection of 2ml/7,5mg (IM) of PGF2α and in the 9th day an injection of 1ml/100μg IM of gonadorelin and an AIFT followed 16h after the second injection of gonadarelin. Furthermore, in the group G2 composed of 20 female buffaloes it was used Ovsynch. The overall pregnancy rates were analyzed by X^2 test. In the Experiment I the pregnancy rate for G1 and G2 were 71.35% and 59.75%, respectively, ($p>0.05$) without any statistic significative difference. In the experiment II, the pregnancy rate for G1 and G2 were 65.5% and 60.0%, respectively, ($p>0.05$) also without any statistic significative difference.The results here obtained are in accordance with literature concerned to the use of steroids and Ovsynch used for synchronize female bovine reported by (Barros&Ereno, 2005). Thus, it can be concluded that the use of protocolls with PRID accomplished with an AIFT for female buffaloes raised n Amazon region showed to be efficients.

EFFECTS OF MANAGEMENT ABOUT PREGNANCY RATE IN THE ARTIFICIAL INSEMINATION PROGRAM

Silva, M.R.[1]; Borges, J.C.[2]; Guimarães, J.D.[3]

[1]DZOMA FCAV-UNESP, -14884-900, Jaboticabal-SP, Brazil, [2]DMVPRA FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil [3]DVT UFV, 36570-000, Viçosa-SP. marcioribsilva@yahoo.com

The objective of this study was to relate management effects in different reproductive seasons in artificial insemination program (AI) about pregnancy rate, in a farm in south pantanal of Brazil. The effects were evaluated: breeding season (BS), number of services, AI time, aspect of mucus, bull and inseminator. The pregnancy rates were obtained in four consecutives BS, fixated in April-June/2003 (BS 1) November-January/2003-2004 (BS 2), April-June/2004 (BS 3), November-January/2004-2005 (BS 4), with 71, 88, 119, 213 heifers, respectively. The results were analyzed in contingency tables and compared by χ^2 test and simple Pearson´s correlations between all effects. It was observed that pregnancy rates of BS 4 (85.45) differed satisfactorily ($p<0.05$) from the previous seasons (69.01, 71.59, 64.71), respectively in chronological order, due the increase in the reposition rate of heifers, and also reproductive management evolution. The number of services not differed ($P>0.05$) between BS, with exception of the third insemination of the BS 2 that differed ($p<0.05$) from excessively, therefore some delayed animals of the first season had been kept in the herd of cattle for second possibility, then the next BS had been rigorous with the discarding of this heifers. The pregnancy rate did not differ ($p>0.05$) how much to AI time, even so registered increase in absolute values when carried through per the morning, suggesting bigger incidence of estrus in the afternoon, due of the possibly be freshness temperature. It did not have effect of the aspect of mucus (clean, dirty and not observed) on the pregnancy rate ($p>0.05$). The pregnancy rate was influenced by inseminator ($p<0.05$), with similar behavior for the effect bull. An experiment to study estrus synchronization in Nelore females demonstrated effect of inseminator in conception rates (MIZUT A et al., Braz. J. Vet. Res. Anim. Sci., v.36:5, 1999) and others works proves the individual bull factors. These two parameters indicate that even so the success of the technique of artificial insemination depends on an adoption of the efficient nutritional, sanitary and reproductive management, over there individual factors as inseminator and bull had relation with the pregnancy rate.

COMPARISON PREGNANCY RATES ACCORDING TO CORPUS LUTEUM SIZE OF INOVULATED EMBRYO RECIPIENTS WITH *Bos indicus* EMBRYOS PRODUCED *IN VITRO*

Borsato, E.A.[1]; Ludwig Jr., H.E.[1]; Silva, D.R.M.[1]; Barreiros, T.R.R.[2]; Seneda, M.M.[3]

[1] Embriogen, Campo Mourão, PR, [2] Faculdades Integrado de Campo Mourão, PR; [3]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051- 990 thalesrigo@grupointegrado.br

The ultrasonography has been applied with success to attended reproduction of bovines. This experiment was carried out in order to compare pregnancy rates of recipient heifers of embryo according to corpus luteum size. Two hundred and three *Bos taurus* x *Bos indicus* heifers were used. In the first group (G-I, n=103), animals with CL detected previously by transrectal ultrasonography (Aloka SSD 500, 5 MHz) received PGF2α - 500 (g IM of Cloprostenol (Sincrocio, Ouro fino, Brazil), and they were kept in heat detection during 72 hours. In the second group (G-II, n=100), heifers received, in a random moment of estrous cycle, an intravaginal device of 1.9 g of P4 (CIDR, Pfizer, Brazil) at the same time as an application of 2mg IM of EB(Estrogin, Farmavet, Brazil). On the eighth day, those devices were removed and animals received 500 μg IM of cloprostenol (Sincrocio, Ouro fino, Brazil). After twenty-four hours all animals received the administration of 1 mg IM of EB. The CL was identified and measured by ultrasonography at 10 and 17 days after the beginning treatment in G-I and G-II**.** All embryo recipients with CL, independent of the size, received embryos produced *in vitro.* Pregnancy diagnosis was done at 38 days after embryo transfer by ultrasonography. This information was analyzed in agreement with the test of the corrected Chi-square of Yates. In the G-I we found 1, 11, 8, 26, 20, 8 and 1 heifers with CL of 12, 15, 16, 18, 20, 22 and 25 mm, respectively. In the G-II we obtained 4, 16, 9, 32 and 18 heifers with CL of 12, 15, 18, 20, 22 mm. Results were pooled in CL with size between 12 to 18 mm and 20 to 25 mm in both groups, and results also showed a significant difference between the groups. The G-I presented 61.3% (46/75) and 37.9% (29/75) ;(P=0.008), respectively with a size between 12 to 18 mm and 20 to 25 mm. In the G-II, there was increased of heifers with CL between 20 to 22 (63.2%, 50/79) than heifers with 12 to 18 mm (36.7%, 29/79); (P=0.001). However, pregnancy rates showed a similarity (P>0.05) in both groups, what results in G-I 36.9% (17/46) and 37.9% (11/29) and in G-II 34.4%(10/29) and 44%(22/50). Although there was no difference on pregnancy rates of embryos produced *in vitro*, there was increased of embryo recipient proportion with bigger corpus luteum when treated with progesterone in embryo transfer at fixed time comparing to recipients cows treated with cloprostenol.

VARIABILITY IN THE NUMBER OF EMBRYOS AT THE OPTIMAL NUMBER OF RECIPIENT DETERMINATION

Beltrame, R.T.[1]; Barioni, L.G.[2]; Quirino, C.R.[3]; Fontes, R.S.[3]

[1]Bolsista Capes, aluno do curso de Pós Graduação em Produção Animal –LMGA – CCTA - UENF, [2] Pesquisador da Embrapa Cerrados Rod. Brasília Fortaleza BR 020 Km 18 Planaltina - DF – Brasil, 73301-970; [3]Professor associado do LMGA – CCTA - UENF. rtbeltrame@terra.com.br

A deterministic analysis assumes that the optimal number of recipient females equals the expected number of embryos to be collected. However, this number changes considerably from donor to donor and for the same donor in different collections. Therefore, using the "ideal" number of recipients, sometimes a surplus and other times a deficit of embryos will occur. A model, implemented in Microsoft Excel v.8.0, was used to predict the number of pregnancies in embryo transfer programs. In that model, the number of embryos collected per donor was generated according a normal distribution with mean equal 6 and different levels of variability. The minimal pregnancy cost was adopted as the criteria to determine the optimal number of recipients. A constant number of donors, six, were considered for each monthly embryo transfer event, during a 36 months period. There was no individualization of the donors, and null covariance was assumed, so that the number of collections and the interval among them for the same donor was unspecified. The first 12 months were discarded to minimize the initial frozen embryo stock effect. The simulation considered that donors were synchronized with a protocol using vaginal implant (CIDR®) and superovulation using FSH (Folltropin®). Synchronization rate of 70%, aptitude rate of 55% and pregnancy rates of 40% and 60%, for frozen and fresh inovulations, resp., were assumed. Four levels of the recipient mean montly cost ($ 6,00; $ 12,00; $ 24,00; $48,00), and four variability levels (standart deviation = 3, 6, 9 e 12) from embryo number collected per donor were tested. 5000 replicates were run in order to get calculate the statistics (mean and distribution) for each case. The deterministic analysis estimated 22,26 as the optimal number of recipients per donor. In this case, the level of montly costs of the recipient did not affect the results. In contrast, the stochastic estimations varied from 21,5 to 26,5, when considering embryo freezing, and 21 to 32,5, when embryos were transferred fresh only. The results of the sensibility analysis allowed observing a reduction of the recipient to donor ratio with the increase of recipient montly cost. This effect was more accentuated as higher variability of standard deviation was considered. Additionally a lower number of pregnancies were obtained with the higher variability in the number of embryos collected. Higher pregnancy numbers and lower variability were obtained when freezing embryos Nevertheless, deterministic estimates can overestimate pregnancy numbers and sub estimate costs. It is concluded that estimates of optimal number of recipients should be based on methods that consider the variability in the number of embryos collected.

MEAN COST FOR THE RECIPIENTS TO EMBRYO TRANSFER

Beltrame, R.T.[1]; Barioni, L.G.[2]; Quirino, C.R.[3]; Fontes, R.S.[3]

[1]Bolsista Capes, aluno do curso de Pós Graduação em Produção Animal –LMGA – CCTA - UENF, [2] Pesquisador da Embrapa Cerrados Rod. Brasília Fortaleza BR 020 Km 18 Planaltina - DF – Brasil, 73301-970; [3]Professor associado do LMGA – CCTA - UENF. rtbeltrame@terra.com.br

Cost forecast for new techniques for animal reproduction (FIV and ET) has received little attention from researchers and extensionists. Such forecast is essential for the evaluation of economic viability and for planning in commercial systems. One of the complexities of that forecast is due to the fact that the costs vary as a function of the time necessary to the attainment of a pregnancy, which follows a probabilistic distribution. Also this distribution varies depending on management and reproductive parameters of performance. Therefore, cost forecast demands models that allow the consideration of the probabilities associated with the event of the recipient getting pregnant in a given point in time. In this study, a dynamic mathematical model, implemented using Stella 8.0 simulation package, was used to simulate the cycle of a group of recipient heifers from purchase up to their pregnancy or descarte. The model considered the following sequence of events: a) Recipient purchase; b) Adaptation and synchronization; c) Embryo transfer; d) inovulation of fresh or unfrozen embryos; e) pregnancy diagnostic. The model allows for reuse of recipient females with negative first inovulation. Negative diagnostic in the second inovulation would result in sale. The recipients in the second cycle go through phases “c” and “e”, in the same way as the recipients in the first cycle. The following reproduction performance parameters were assumed: proportion of cycling females = 0,7; synchronization rate = 0,7; aptitude rate = 0,55; preganancy rate (from fresh inovulation) = 0,6 and (from unfrozen embryos) = 0,4. Market prices of May/2005 were used. The montly costs associated with each phase were estimated as (R$): 1) heifer purchase = 750,00; 2) Adaptation = 54,21; 3) non-inovulated females = 26,33; 4) on transfer = 48,33; 5) on diagnostic = 28,47; 6) sale of non-pregnant heifer = 600,00. The total cost of recipient females was calculated from a monthly cash flow budget, calculated as the sum of product of the probability of the recipient to be in the i^{th} phase and the associated costs of that phase. The discarded recipients were taken as revenue. The future value of the cash flow was calculate for the end of the period taken (24 months) using a monthly interest rate of 0.5%. The model predicts, using the parameters informed above, that around 83% of the females would get pregnant up to the end of the simulated period. The mean cost for the recipients in the program was estimated as R$ 1.212,80 (including pregnant and non-pregnant females). The cost estimated for pregnant females was R$ 1.344,81. Those estimatives disconsider the costs of the embryos for the program.

GESTATION RATE OF HEIFERS FED MEGALAC OR LINSEED GRAIN FOLLOWING TRANSFER OF EMBRYO COLLECTED FROM DAIRY COWS FED MEGALAC OR LINSEED GRAIN

Cavalieri, F.L.B.[1]; Santos, G.T.[2]; Petit, H.[3]; Morgan, J.[4]; Rigolon, L.P.R.[1,2]; Ramos, F.S.R.[1]; Capovilla, L.C.T.[1,2]; Albuquerque, K.[2]

[1] Professores do Curso de Medicina Veterinária do Cesumar; [2] Curso de pós-graduação em Zootecnia da Universidade Estadual de Maringá; [3] AAFC, Dairy and Swine R&D Centre, Lennoxville, QC, Canada; [4] Kemptville College, Kemptiville, ON, Canada. fabiobim@cesumar.br

The experiment was done in the School of Agricultura Kemptiville belonging to the University of Guelph - Ontário – Canada. Seventy five heifers of the was used with avereged weight of 350 kg divided in two treatments: T1 – Megalac® (39 animals) and T2 - Linseed in grain (36 animals). The diets were supplied 8 weeks before the syncronization of the estrus until complete 50 days of gestation. The inovulation of the embryos was accomplished seven days after the estrus with frozen embryos from dairy cows fed either Megalac or Linseed in grain. The experimental designed was casual totally and the animals were distributed in a factorial outline 2 x 2, being two treatments (Megalac® or Linseed grain) and two types of embryos in agreement with the animals donors' feeding (Megalac® or Linseed grain). For the variable gestation rate it was used the distribution of binomial probability and the function of connection logistics, though of methodology of widespread lineal models (Nelder and Wedderburn, 1972), being used the software GLIM 4.0. There was not treatment effect ($p>0.05$) in the pregnant rate of inovulation of the embryos in the animals fed with Linseed in grain or Megalac® (T1: 38,89 e T2, 46,15). However the animals that received frozen embryos of the cows fed with Linseed presented ($p <0.05$) an increase in the pregnant rate (58.82%) when comparing to the donors' received frozen embryos fed with Megalac® (29.27%). This way we can end that there was not treatment effect the pregnant rate in the inovulation, though the collected embryos of having fed with Linseed could be more resistant to the freezing than those collected of cows fed with Megalac®.

EFFECTS OF TWO SOURCES OF FAT (MEGALAC® OR LINSEED GRAIN) IN THE DIET IN THE PRODUCTION OF THE EMBRYOS IN DAIRY COW

Cavalieri, F.L.B.[1]; Santos, G.T.[2]; Petit, H.[3]; Morgan, J.[4]; Rigolon, L.P.R.[1,2]; Ramos, F.S.R.[1]; Capovilla, L.C.T.[1,2]; Albuquerque, K.[2]

[1] Professores do Curso de Medicina Veterinária do Cesumar; [2] Curso de pós-graduação em Zootecnia da Universidade Estadual de Maringá; [3] AAFC, Dairy and Swine R&D Centre, Lennoxville, QC, Canada; [4] Kemptville College, Kemptiville, ON, Canada. fabiobim@cesumar.br

The experiment was done in the School of Agricultura Kemptiville belonging to the University of Guelph - Ontário – Canada, aiming at to evaluate the effect of two fat sources in the production of embryos in cows milk. Thirty cows used divided in two treatments with 15 repetitions each: T1 – Megalac® (omega 6) and T2 - Linseed in grain (omega 3). The diets were supplied twice a day starting from the 21º post parturition day, and 80 days after the animals were synchronized with one it implants intravaginal (CIDRr - InterAg, Hamilton, New Zealand) and the application of 3 mg estradiol 17ß 24 hours after the insert of the it implants. Four days after the animals were superovulation with 400 mg of FSH (Folltropinr - Vetrepharm, Canada) in two decreasing daily doses for 5 days. The animals were inseminated 12, 24 and 36 hours after the detection of the estrus with semen of a bull of the Holstein breed and the flushing of the embryos was accomplished by the non surgical method (open), seven days after the estrus, accomplishing two flushing in each cow in an interval of 45 days. Five animals of each treatment were observed twice a week, starting from the beginning of the experiment until sincronization protocol, evaluating number of small follicles (<5 mm), medium (5,0 to 9,0 mm) and larger (> 10 mm). The experimental designed was ramdomized, and, for to the variables number of follicles and embryos the function of distribution of probability was admitted Poisson and function of logarithmic connection, through the methodology of widespread lineal models (Nelder and Wedderburn, 1972), being used the software GLIM 4.0. There was not treatment effect (p>0.05) in the number of small follicles (T1:1,79 e T2: 1,61), medium (T1:1,32 e T2:1,55) and larger (T1:0,27 e T2:0,31) as well as in the number of total embryo (T1: 5,65 e T2: 6,57) transferable (T1: 3,42 e T2: 2,38) and degenerated embryos ((T1: 1,07 e T2: 1,42).This way we can end that the donors' feeding and or receiving with Megalac (ômega 6) and or Linseed in grain (ômega 3) it doesn't alter the number of follicles and embryo production.

DETERMINATION OF ANCESTRAL PROPORTIONS IN SYNTHETIC BOVINE RACES USING DNA MARKERS: APPLICATION TO INDIVIDUAL GIROLANDO ANIMALS

Bicalho, H.M.S.; Pimenta, C.G.; Mendes, I.K.P.; Pena, H.B.; Lopes, C.; Queiroz, E.M.; Rocha, J.L.F.; Pena, S.D.J.

Gene/Genealógica Central de GenoTipagem de Animais Ltda., Belo Horizonte-MG.
genegenealogica@genealogica.com.br

The subspecies *indicus* (Zebu) and *taurus* (taurine) of *Bos primigenius* diverged more than 600,000 years ago. With the passage of time their genomes accumulated differences that can be evaluated by DNA tests, especially using microsatellites. In the first half of the 20th century several synthetic races were developed by crossing Zebu and taurine cattle, as exemplified today in Brazil by the races Girolando (Gir X Holstein), Brangus (Brahman X Angus) and Simbrasil (Guzerat X Simmenthal). All these races have as standard proportions of 3/8 Zebu and 5/8 taurine. Certainly it is of considerable interest the determination of the ancestral proportions in individual animals for purposes of registration and breeding. The power of a set of nine microsatellites for the analysis of ancestry in Girolando animals was investigated. This set is recommended by ISAG (International Society of Animal Genetics) and officially adopted by the Ministry of Agriculture for parentage testing in Brazil. The genotypes of 50 Gir animals and 50 Holstein animals were obtained for the following microsatellites: BM1824, BM2113, ETH10, ETH225, INRA23, SPS115, TGLA122, TGLA126 e TGLA227. A group of 20 Girolando animals was also typed for which there was genealogical information about the admixture proportions of genes of Gir and Holstein. There were very significant allele frequency differences at most loci. The genotypes were analyzed by the program Structure 2.0 that uses a Bayesian algorithm to perform k-means clustering. Based on the genotypes of the Gir and Holstein animals, the software attributed to each of the 20 Girolando animals a genomic proportion of Zebu and taurine. The results obtained were compared with the genealogical data of the animals, producing a highly significant correlation coefficient of 0.84 ($P < 0.0001$). Conclusion: the panel of 9 microsatellites was capable of estimating with excellent fidelity the ancestral proportions of Gir and Holstein in individual Girolando animals, demonstrating the high degree of accuracy of the analysis. The reliability of this test could be improved even further by the inclusion of additional genomic markers.

PREGNANCY RATES IN EMBRYO RECIPIENTS TREATED WITH PROGESTERONE RELEASING DEVICES CONTAINING TWO DIFFERENT PROGESTERONE CONCENTRATIONS

Peres, L. C.[1,2]; Pincinato, D.[1,2]; Balla, E.[1,2]; Tribulo, R.[2]; Bó, G.A.[1,2]

[1]Universidad Nacional de Córdoba, Argentina. [2]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

An experiment was designed to compare pregnancy rates in embryo recipients treated with progesterone (P4) releasing devices Triu-B (Triu-B, Biogénesis, Argentina) containing 0.5 or 1 g of P4 and transferred at a fixed time (FTET). Non-lactating and cycling (with a CL or a follicle >10 mm and uterine tone determined by rectal palpation) *Bos taurus x Bos indicus* cows, with a body condition score of 3 to 4 (1 to 5 scale) were used. On Day 0, all cows received 2 mg of estradiol benzoate (EB, Bioestrogen, Biogénesis) and were randomly divided to receive a Triu-B device containing either 0.5 g of P4 (Triu-B Monodosis) or a Triu-B containing 1 g of P4. On Day 5, all cows received 400 UI eCG (Novormón 5000, Syntex, Argentina) and 150μg D (+) cloprostenol i.m. (Enzaprost-DC, Biogénesis). Triu-B devices were removed on Day 8 and cows received 1 mg de EB on Day 9. On Day 17, recipients were examined by rectal palpation and those with a CL received fresh embryos by non-surgical transfer in the ipsilateral horn to the CL. The embryos used were 41 Grade 1 and 8 Grade 2 (IETS). Pregnancy diagnosis was performed 70 days after FTET by ultrasonography and data was analyzed by logistic regression. Pregnancy rates were not influenced by embryo grade (P=0.4) or body condition score (P=0.1). Furthermore, no differences were detected between groups in the proportions of recipients transferred/ treated (Triu-B 0.5 g: 23/30, 76.7% vs Triu-B 1g: 26/30, 86.7%; P=0.31), recipients pregnant/transferred (Triu-B 0.5 g: 15/23, 65.2% vs Triu-B 1 g: 12/26, 46.2%; P=0.13) and recipients pregnant/treated (Triu-B 0.5 g: 15/30, 50.0% vs Triu-B 1 g: 12/30, 40.0%; P=0.43). It was concluded that both devices are equally efficacious in FTET synchronization programs in *Bos indicus* × *Bos taurus* recipients. More experiments are need to confirm this results.

PREGNANCY RATES IN RECIPIENT COWS TREATED WITH PROGESTERONE VAGINAL DEVICES AND INDUCED TO OVULATE WITH ESTRADIOL BENZOATE GIVEN AT THE TIME OF DEVICE REMOVAL OR 24 H LATER

Peres, L.C.[1,3]; Pincinato, D.[1,3]; Balla, E.[1,3]; Maraña Peña, D.[1,3]; Borges, L.F.K.[2]; Cutaia, L.[3]; Bó, G.A.[1,3]

[1]Universidad Nacional de Córdoba, Argentina, [2]Universidade Federal de Santa María, Brasil, [3]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

An experiment was designed to compare pregnancy rates in cows treated with progesterone releasing devices (DIB, Syntex, Argentina) plus EB(Syntex) and eCG (Novormon, Syntex), and induced to ovulate with EB given at device removal or 24h later. Non-lactating, cycling, crossbred Zebu cows (n=478), with a body condition score between 2.5 to 3.5 (1 to 5 scale) were used. Data was obtained in 5 replicates. All cows were treated with a DIB device and 2mg EB i.m., on Day 0 and 400IU of eCG i.m. plus 150µg D(+)cloprostenol i.m. (Ciclase, Syntex), on Day 5. On Day 8, DIB devices were removed and cows were randomly divided to receive 1mg EB i.m. at the time of DIB removal (EB0) or 24h later (Day 9; EB24). Recipients were observed for signs of estrus from Day 8 to 13. On Day 16, all recipients with >1 CL, or a single CL with an area >256mm^2, measured by ultrasonography, were transferred at a fixed time (FTET) with fresh or frozen/thawed embryos on Day 16 (EB0) or Day 17 (EB24). The embryos used were 253 Grade 1, 65 Grade 2 and 33 Grade 3 (IETS). Ovarian ultrasonography was performed on Day 0, to determine ovarian status (only cows with a CL or a follicle >10mm and uterine tone were used) and 30 days after TETF, to determine pregnancy status (100 Falco Vet., Pie Medical, 8,0 MHz transducer). Quantitative data was analyzed by ANOVA and qualitative data was analyzed by logistic regression. The variables fresh or frozen embryos, technician, embryo stage and CL area did not affect pregnancy rates (P>0.05). However, embryo quality tended (P=0.06) to influence pregnancy rates (Grade 1: 152/253, 60.1%, Grade 2 32/65, 49.2%, Grade 3 11/33, 33.3%). The interval from DIB removal to estrus was shorter (P=0.0001) for the recipients in Group EB0 (26.5±0.6h) than in those in Group EB24 (39.8±0.8h). Nevertheless, pregnancy rates were not different (P=0.6) between recipients seen (EB0: 83/137; 60.6% e EB24: 60/117 51.3%) or not seen in estrus (EB0: 27/43; 62.8% e EB24 25/54; 46.3%). The rate of recipients transferred/treated was not different (P=0.5) among groups (EB0: 180/241; 74.7% and EB24: 171/237; 72.2%). Conversely, the rate of recipients pregnant/transferred and pregnant/treated were higher (P=0.03) in Group EB0 (110/180; 61.1% and 110/241; 45.6%) than in Group EB24 (85/171; 49.7% and 85/237; 35.9%), respectively. It was concluded that the use of EB at device removal could reduce the number of trips through the chute for treatments and possibly improve pregnancy rates in a FTET program.

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PREGNANCY RATES IN EMBRYO RECIPIENTS TREATED WITH NORGESTOMET EAR IMPLANTS AND ESTRADIOL BENZOATE OR DIFFERENT DOSAGES OF ESTRADIOL VALERATE

Peres, L.C.[1,3]; Pincinato, D.[1,3]; Maraña Peña, D.[1,3] ; Borges, L.F.K.[2]; Tribulo, R.[1,3]; Cutaia L.[3]; Bó G. A.[1,3]

[1]Universidad Nacional de Córdoba, Argentina, [3] Universidade Federal de Santa María, Brasil, [3]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

An experiment was designed to evaluate pregnancy rates in embryo recipient cows treated with norgestomet ear implants (Crestar, Intervet, Brasil) plus estradiol benzoate (EB; Syntex, Argentina) or different dosages of estradiol valerate (EV). Non-lactating multiparous crossbred Zebu cows (n=216) with body condition score between 3.5 and 4.5 (1 to 5 scale) were used. On Day 0 all cows received a Crestar ear implant and were randomly allocated into 1 of 4 treatment groups to receive, at the same time, 2 mg EB plus 50 mg of progesterone (Laboratorio Rio de Janeiro, Argentina), 5 mg EV plus 3 mg norgestomet (2 ml) or 2 mg EV and 1,2 mg norgestomet (0,8 ml). All cows received 400 UI eCG (Folligon 5000, Intervet, Brasil) and 150 ìg D (+) cloprostenol i.m. (Preloban, Intervet) on Day 5 and Crestar implants were removed on Day 8 (Groups EB2mg/8 d and EV2mg/8d) or Day 9 (Groups EV 2mg/9 d and EV 5mg/9 d). All cows received 1 mg de EB i.m. 24 h after implant removal and were observed for signs of estrus from Day 8 to 13. On Day 16, all recipients were examined by ultrasonography and those with >1 CL, or a single CL with an area >256 mm^2 were selected to receive frozen/thawed embryos on Day 17 (EB2mg/8 d and EV 2mg/8 d) or on Day 18 (EV 2mg/9 d and EV 5mg/9 d). The embryos used were 145 Grade 1 frozen/thawed embryos. . The interval from Crestar removal to estrus was 47,1±0,8 h and was not different among groups (P=0.4). Furthermore, the number of CL was not different (P=0,8) among groups (EB 2mg/8 d 1,4±0,1; EV 2mg/8 d 1,3±0,1; EV 2mg/9 d 1,2±0.1; EV 5 mg/9 d 1,5±0,1). Pregnancy rates were not influenced by embryo stage , CL area or if recipients were observed or not observed in estrus (P>0,4). Although the rate of recipients transferred/pregnant was not different (P=0,5) among groups the rate of recipients pregnant/transferred was higher (P=0,004) in Groups EB 2mg/8 d (22/35; 62,8%), EV 2mg/8 d (26/40 65,0%) and EV 5mg/9 d 18/32 56,3%) than in Group EV 2mg/9 d (12/37 31,5%). Consequently, the rate of recipient pregnant/treated was also higher (P=0,02) in groups EB 2mg/8 d (22/50; 44,0%); EV 2mg/8 d (26/53; 49,1%) and EV 5mg/9 d (18/54 33,3%) than in Group EV 2 mg/9 d (12/53 22,6%). Preliminary data indicated that the reducing the EV dosage to 2 mg resulted in comparable pregnancy rates when the Crestar was removed on Day 8 but not when Crestar was removed on Day 9.

EVALUATION OF SECOND UTERINE FLUSHING ON EMBRYO RECOVERY RATE IN NELORE COWS – PRELIMINARY RESULTS

Barrreiros, T.R.R.[1]; Bezerra, G.A.[2]; Azevedo, J.R.[3]; Seneda, M.M.[4]

[1]Faculdades Integrado de Campo Mourão/PR, [2]Médico Veterinário Autônomo, [3]Mestrando em Reprodução Animal, DZO, UEM, [4]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051-990 thalesrigo@grupointegrado.br

The aim of this trial was to analyze the additional flushing on embryo recovery. Five cyclic Nelore donor cows were submitted the two sessions of multiple ovulation and embryo transfer. In the first session, the cows received in random stages of the estrus cycle, an intravaginal progesterone device (Cronipress, Biogenisis, Argentina) and 2.5 mg intramuscular (IM) of estradiol benzoate (Estrogin, Farmavet, Brasil). Five days later, was administered 250 IU of FSH (Pluset, Calier, Espanha), in 8 decrescent IM doses and twice a day, in the next four days. Forty and eight hours a after the early FSH treatment, was applied 150 μg, IM, of cloprostenol (Croniben, Biogenesis, Brasil) and 12 hours later withdraw the progesterone device. Artificial insemination was performed 12 and 24 hours after estrus detection. On the second session the donors received similar treatment, delay withdraw the progesterone device 24 hours after cloprostenol administration, plus a 1500 IU of hCG (Vetecor, Calier, Espanha) in the morning of nine day of the treatment. The animals were inseminated 12 and 24 hs after hCG administration. The embryo recovery was made sixteen days after the early of treatment, with 1 L DPBS (DPBS, Nutricell, Brasil) and a Foley catheter positioned on uterine body. Twenty four hours later, was made the second uterine flushing. The embryos classified as grade I, II, III and IV - IETS. At the end of this trial, the results will be analyse by Tukey test. On the first session was obtained 60 structures, 43 viable embryos, (average of 12 and 8.6). On the second session recovered 52 structures and 31 viable embryos (average 10.4 and 6.2). On the first session, the additional uterine flushing results in 9 structures and 7 viable embryos from two donnors and 2 viable embryos on the second session, from one cow. Results showed that when two uterine flushing was performed, the average of recovered structures increased from 8.6 to 10 viable embryos on the first session and from 6.2 to 6.6 viable embryos on the second session. The preliminaries results showed additional uterine flushing, 24 hours after conventional procedure, may increased the embryo recovery rates in cows in estrus detection protocol.

EVALUATION OF *CORPUS LUTEUM*, UTERINE CONTRACTILITY AND PROGESTERONE AND ESTRADIOL PLASMATIC CONCENTRATIONS IN RECIPIENTS HEIFERS IN THE DAY OF EMBRYO TRANSFER

Leal, L.S.[1]; Oba, E.[1]; Fernandes, C.A.C.[1]; Sá Filho, O.G.[1]

[1]Department of Animal Reproduction and Veterinary Radiology – FMVZ – UNESP – Botucatu - SP 18618-000 – Brazil, lu_s_leal@yahoo.com.br

The aims of the present study were to evaluate the size of *corpus luteum* (CL) by rectal palpation and by ultrasound and uterine contractility and to determine progesterone (P_4) and estradiol ($17ßE_2$) plasmatic concentrations in recipients, in the day of embryo transfer. As recipients 60 crossbred heifers were used. The pregnancy rate of recipients according to the CL size by rectal palpation was 70.00% (small), 38.89% (medium) e 60.00% (big). Ultrasonographic evaluation detected 50.88% of the CL with a cystic cavity. The average diameter of the CL was 17.82 ± 6.26 mm and of cystic cavity was 6.76 ± 4.19 mm. The diameter of the CL and of cystic cavity did not interfere with pregnancy rates. The mean volume of the CL was 4.36 ± 9.09 cm^3, of cystic cavity was 0.40 ± 0.97 cm^3 and of luteal mass was 4.16 ± 8.42 cm^3. The volume of the CL, of cystic cavity and of luteal mass did not influence the pregnancy rates. In the day of embryo inovulation progesterone and estradiol plasmatic concentrations were respectively 4.91 ± 2.97 ng/mL and 4.45 ± 6.03 pg/mL in average. There was no statistics difference between progesterone and estradiol concentrations for pregnant and non-pregnant recipients. Forty-nine (81.67%) of the 60 recipients presented flaccid uterus (grade 1) and 11 (18.33%) had uterus in intermediate stage (grade 2). Those presented pregnancy rates of 47.62% and 81.82%, respectively ($p<0.05$ – Qui-square Test), concluding that recipients whose uterus had been classified as grade 2 had presented the best results of gestation.

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COMPARISON OF PREGNANCY RATES IN HEIFERS WITH COMPACT OR CAVITARY CORPUS LUTEUM TRANSFERRED WITH IN VITRO PRODUCED EMBRYOS

Borsato, E.A.[1]; Ludwig Jr., H.E.[1]; Silva, D.R.M.[1]; Barreiros, T.R.R.[2]; Seneda, M.M.[3]

[1] Embriogen, Campo Mourão, PR, [2] Faculdades Integrado de Campo Mourão, PR; [3]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051- 990. thalesrigo@grupointegrado.br

The aim of this work was to evaluate a number of animals ready to embryo transfer and pregnancy rates in bovine embryo recipients with compact and cavitary corpus luteum. Two hundred and three crossbred (*Bos taurus x Bos indicus*) heifers were divided in two groups. In the first group (G-PGF, n=103), the animals received 500 μg de Cloprostenol IM (Sincrocio, Ouro fino, Brasil) after CL identification by ultrasonography (Aloka SSD 500, 5 MHz). After that, heifers were detected for heat the next 72 hours. The animals from second group (G-P4, n=100) received 1.9g implant of intravaginal progesterone (CIDR, Pfizer, Brasil) and 2 mg estradiol benzoate (Estrogin, Farmavet, Brasil) IM, in random stages from estrus cycle. On eight day (D8) the implants were removed and the animals received an IM of 500 μg de Cloprostenol IM. After 24h all animals received 1 mg estradiol benzoate IM. The CL was identified and evaluated at 10 and 17 days after the beginning treatment in G-PGF and G-P4 respectively. All the heifers with CL received IVP embryo. The pregnancy diagnosis was made by transrectal ultrasonography after 45 days. The results were analyzed by Chi-square. There were no significant difference in number of heifers ready to embryo transfer in G-PGF and G-P4, respectively 72.8% (75/103) e 79% (79/100); (P>0.05). Twenty one percent (16/75) had cavitary CL in G-PGF and 22.7% (18/79) in G-P4 (P>0.05). The pregnancy rates did not result in significant difference 37.3% (28/75) for G-PGF and 40.5% (32/79) for G-P4 (P>0.05). Among all embryo recipients with a cavitary or compact CL there was no difference on pregnancy rates (41.1% 14/34 and 38.3% 46/120, respectively, P>0.05). The treatments did not affect a number of heifers ready to embryo transfer and pregnancy rates for IVP embryos. These results show that there is a similarity on cavitary CL embryo recipient pregnancy rates comparing to compact CL ones regardless to protocol treatment group. Furthermore, there was no difference on recipient usage and pregnancy rates, after cloprostenol and intravaginal progesterone treatment. However this is a necessity of CL evaluation by rectal palpation or ultrasonography, before cloprostenol treatment. It shows an easily management when fixed time embryo transfer is used.

EFFECT OF MOMENT OF PGF2α ADMINISTRATION AND eCG DOSE IN SYNCHRONIZATION OF OVULATION FOR FIXED-TIME EMBRYO TRANSFER IN HEIFERS

Rezende, C.R.[1]; Nasser, L.F.[1,2]; Rezende, L.F.[1]; Nunez, M.M.[1]; Vieira, M.P.[1]; Ayres, H.[2]; Sá Filho, M.F.[2]; Gimenes, L.U.[2]; Baruselli, P.S.[2]

[1]FIRMASA, Campo Grande-MS, Brasil. [2]Departamento de Reprodução Animal, FMVZ/ USP, São Paulo/SP, Brasil. barusell@usp.br

The aim was to compare the efficacy of $PGF_{2\alpha}$ utilization at the beginning of treatment associated to different eCG doses (300 or 400 UI) at device removal (fatorial 2x2) during fixed-time embryo transfer protocol (FTET) in *Bos taurus* x *Bos indicus* heifers. All heifers (n=378) presented good body condition (>3,0, 1 a 5 scale), and were previously examined by ultrasongraphy (US) for cyclicity (CL presence) and assigned to four groups according to PGF2α administration or not at insertion and to 300 or 400 UI of eCG administration (Folligon®, Intervet) at intravaginal progesterone releasing device (IPD, Cronipres®, Biogenesis, Brasil) removal. At random stage of the estrous cycle (Day 0), the females received 2mg of estradiol benzoate, i.m. (Ric-BE®, Tecnopec, Brasil) and an IPD. At Day 8, the IPD was withdraw and PGF2α (Croniben®, Biogenesis, Brasil) was administered plus 0.5mg of estradiol cypionate i.m. (ECP, Pfizer, Brasil). The recipients were submitted to US at FTET on Day 17, and the able ones (CL ≥ 18 mm of diameter) received an *in vitro* embryo. The pregnancy diagnosis was performed by US 30 days after FTET. The selection rates (SR; transferred/treated); conception rate (CONC), pregnancy rate (PREN) and the number of CL at FTET (NCL) were analyzed by SAS. No interactions were observed being the results of the Groups treated with vs. without PGF and the Groups treated with 300 vs. 400 eCG were, respectively: SR [92.7% (177/191) vs. 92.5% (165/187) e 93.6% (176/188) vs. 91.6% (174/190)], **CONC** [48.6% (86/177) vs. 49.1% (81/165) e 45.4% (80/176) vs. 50.0 (87/174)], **PREN** [43.3% (81/187) vs. 45.0% (86/191) e 42.6% (80/188) vs. 45.7% (87/190)], **NCL** (1.15±0.03 vs. 1.11±0.04 e 1.11±0.04 vs. 1.15±0.03; P>0.05). The PGF2α administration on Day 0 did not alter the variables analyzed (p>0.05). The data suggest that 300 UI of eCG administration promote similar efficiency than 400 UI (p>0.05) and it can be used in FTET protocol in *Bos taurus* x *indicus* heifers.

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EFFECT OF LUTEOLISIS TO ESTRUS INTERVAL , PERIOD OF THE YEAR AND FSH DOSE ON EMBRYO PRODUCTION IN BEEF CATTLE

Brauner, C.C.[1]; Bonotto, A.L.M.[2]; Pimentel, M.A.[3]; Pimentel, C.A.[4]

[1] Aluno bolsista CnPq de Mestrado do PPG do Departamento de Zootecnia, FAEM, UFPel. Departamento de Zootecnia FAEM/UFPel, caixa postal 354, 96010-970. Pelotas-RS. AC. Prof. Marcelo Pimentel. cassiocb@brturbo.com [2] Médica Veterinária Autônoma. [3] Professor Adjunto do Departamento de Zootecnia, FAEM, UFPel. [4] Pesquisador do CnPq.

The objective of this study was to evaluate the effect of the period of the year, FSH dose and PGF2α to estrus interval on embryo production in beef cattle. Data referring to 90 flushes in Aberdeen Angus cows of a commercial establishment located in Maçambará city, west region of the state of the RS, during the years of 2002 to 2004, were analyzed. The fixed factors analyzed were four periods of the year (January to March; April to June; July to September and October to December), four doses of FSH (250; 287; 300 and 325 U.I.), and three intervals between administration of PGF2α and estrus behavior (28 to 36 h; 36 to 44 h and 44 to 52 h). The variables analyzed were: total structures (ET), viable structures (EV) and not viable structures (NV), in agreement with IETS standards (1998). Data were submitted to the analysis of variance (ANOVA – GLM), in the program NCSS 6.0 (1996). The ET was lower ($P<0.05$) in interval 44 to 52 h, with 9.1±1.6; 9.0±0.7 and 5.8±1.0, for the three intervals etudied, respectively. The period of the year and dose of FSH did not present effect on ET. The EV was not affected by the fixed effects studied. The NV was greater ($P<0.05$) in interval 36 to 44 h, with 2.0±1,1; 3.2±0.5 and 1.1±0.7 for the three intervals etudied, respectively. The period of the year and dose of FSH did not present effect on NV. We concluded that the increase interval between the application of $PGF_2\alpha$ and the estrus manifestation has negative effect on the production of total structures recoveries, however epoch of the year and dose of FSH do not show effect on ET, EV and NV.

FOLLICULAR DYNAMICS IN HEIFERS AND COWS (*Bos indicus*) TREATED WITH NEW OR PREVIOUSLY USED INTRAVAGINAL PROGESTERONE DEVICE ASSOCIATED OR NO TO INJECTION OF PROGESTERONE

Martins, C.M.[1]; Castricini, E.S.C.[1]; Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ – USP, São Paulo – SP, 05508-000, Brasil.
barusell@usp.br

A total of 30 Nelore heifers and 30 Nelore lactating cows (70-90 days postpartum) were homogeneously assigned in three experimental groups (newDIB, 3thuseDIB e 3thuseDIB+P4). At random stages of the estrous (Day 0) the females were treated with 2mg of estradiol benzoate (EB) i.m. (RIC-BE®, Syntex, Argentina) plus intravaginal progesterone releasing device (DIB®, Syntex, Argentina) new (n= 10 heifers and 10 cows) or used (previously used for 16 days; n= 10 heifers and 10 cows). The animals of the Group 3thuseDIB+P4 (n= 10 heifers and 10 cows) received a used DIB plus 2mg of BE associated to 50 mg of P4 i.m (P4; Progesterone 20mg/ml, Syntex, Argentina) on Day 0. On Day 8, the devices were withdraw and 150mg of D-cloprostenol i.m. (PGF2α; Prolise®, Syntex, Argentina) were administered and 1mg of EB i.m. on Day 9. The follicular dynamic was evaluated by ultrasonographic scanners performed every 24 hours from Day 0 to Day 8 and every 12 hours from Day 9 to Day 12. Data were analyzed by Anova, Chi-square and Bartlett tests. No interaction was observed between cows and heifers in the analyzed variables and the results for newDIB, 3thuseDIB e 3thuseDIB+P4 were, respectively: day of emergence of the new follicular wave (3.1±0.22^{b,x}, 2.8±0.22^{b,x} and 4.2±0.08^{a,y} days; $p<0.05$), maximum diameter of the dominant follicle (1.42±0.04, 1.33±0.03 and 1.32±0.03 cm; $p> 0.05$), ovulation rates [95.0% (19/20); 95.0% (19/20) and 80.0% (16/20); $p > 0.05$] and moment of ovulation after device removal (68.8±2.2, 71.4±2.2 and 66.4±2.1h; $p >0.05$). The Group 3thuseDIB+P4 delayed the emergence of new follicular wave, however the same was more synchronized than others groups. The previously used devices (3thuseDIB) presented equal response to new DIB in the synchronization of ovulation in Nelore cows and heifers.

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FIXED-TIME EMBRYO TRANSFER IN *Bos taurus* x *Bos indicus* HEIFERS TREATED WITH eCG AT MOMENT INTRAVAGINAL PROGESTERONE RELEASING DEVICE WITHDRAWAL AND GnRH AT EMBRYO TRANSFER

Nasser, L.F.[1,2]; Rezende, L.F.[1]; Rezende, C.R.[1]; Vieira, M.P.[1]; Wisnesck, C.A.[1]; Sá Filho, M.F.[2]; Gimenes, L.U.[2]; Baruselli, P.S.[2]

[1]FIRMASA, Campo Grande-MS, Brasil. [2]Departamento de Reprodução Animal, FMVZ/ USP, São Paulo/SP, Brasil. barusell@usp.br

In the present study the effect of equine chorionic gonadotropin (eCG) and/or of a GnRH analog in fixed-time embryo transfer (FTET) in *Bos taurus* x *Bos indicus* heifers was evaluated. All females (n=239) presented good body condition score (>3,0 – 1 to 5 scale) and were previously examined by ultrasonography (US; *corpus luteum* presence) and assigned in four groups according to eCG administration or not (400UI, Folligon, Intervet, Brazil) at intravaginal progesterone releasing device (IPD; CIDR, Pfizer, Brazil) or to GnRH, i.m., (Conceptal, Intervet, Brazil) at FTET (2x2 factorial). In the beginning of treatment (Day 0) heifers received a CIDR and 2mg of estradiol benzoate i.m. (EB; Estrogin, Farmavet, Brazil) plus ½ dose of PGF2α (12.5 mg of dinoprost, Lutalise, Pfizer, Brazil). On Day 8, CIDR was removed, 0,5mg of estradiol cypionate (ECP, Pfizer, Brazil) and 1/2 dose of PGF2α were administered. Control Group received no additional treatment, while in eCG Group eCG was administered on Day 8 and GnRH Group was treated with GnRH at FTET. The eCG+GnRH Group received both treatments. On Day 16, recipients were evaluated by US (CL ≥ 18 mm diameter) and received one *in vitro* embryo. Data were analyzed in SAS program. No interactions were observed between treatments. The main effects of the recipients treated with vs. without eCG and treated with vs. without GnRH, were, respectively: transferred/ treated rate [89.9% (107/119) vs. 85.8% (103/120) and 87.3% (103/118) vs. 88.4% (107/121)]; pregnant/ transferred rate [49.5% (53/107) vs. 51.5% (53/103) and 51.5% (53/103) vs. 49.5% (53/107)] and pregnant/ treated rate [44,5% (53/119) vs. 44.2% (53/120) e 44.9% (53/118) vs. 43.8% (53/121)]. There were no effects of treatment in variables analyzed (p>0,05), possibly due to excellent nutritional conditions and cyclicity in the beginning of treatment. In conclusion, in the specific conditions of this experiment, eCG or GnRH treatment did not promote improvement in the FTET protocols efficiency in heifers.

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EFFECT OF THE NUMBER OF NORGESTOMET IMPLANT AND OF THE TIME OF OVULATION INDUCER ADMINISTRATION IN SUPERSTIMULATED HOLSTEIN COWS

Rodrigues, C.[1]; Mancilha, R.F.[1]; Reis, E.L.[2]; Ayres, H.[2]; Gimenes, L.U.[2]; Sá Filho, M.F.[2]; Baruselli, P.S.[2]

[1]Clínica Samvet, São Carlos-SP, [2] Dep. Reprodução Animal, FMVZ/USP, São Paulo/ SP, Brasil. barusell@usp.br

A total of 40 Holstein cows (>39L/day) were assigned in four groups: 1CrestarGnRH48; 1CrestarGnRH60; 2CrestarGnRH48 and 2CrestarGnRH60 (factorial 2x2). On Day 0, the females received one or two auricular Norgestomet implant (Crestar®, Intervet, Brazil) and 3mg of estradiol benzoate plus progesterone i,.m.(Index Farmacêutica, Brazil). Superstimulation was performed with 400 UI of FSHp (Pluset®, Calier, Brazil) in 8 decreasing doses each 12h, from Day 4. On Day 6, the females received two doses (morning and afternoon) of PGF2α (Preloban®, Intervet, Brazil) and the implants were withdraw 36h after first PGF2α injection. The GnRH i.m.(250μg, Fertagyl®, Intervet, Brazil) was administered 48h (GnRH48) or 60h (GnRH60) after the first PGF2α. Inseminations were performed 12 and 24h after GnRH, been the sires homogeneity assigned between the treatments. The embryo recovery were performance on Day 15. The data were analyzed by Anova and Chi-square tests. No interaction was observed between the variables. The main effects for treatments with 1Crestar vs 2Crestar and GnRH48 vs GnRH60 were, respectively: number of CL on Day 15 (10.5±2.1 vs 9.6±1.8 and 10.2±2.1 vs 9.9±1.8; $p>0.05$), number of follicles >8 mm on Day 15 (2.6±0.4 vs 2.7±0.5 and 3.5 ± 0.5^{a} vs 1.7 ± 0.3^{b}; $a\neq b$ $p<0.05$), total structures (8.4±2.3 vs 8.5±2.0 and 8.3±2.3 vs 8.6±2.0. $p>0.05$), transferable embryos (3.0±0.9 vs 3.8±1.2 and 2.7±0.8 vs 4.2±1.3; $p>0.05$) e suitable for freezing embryos (1.5±0.5 vs 3.0±1.0 and 1.6±0.6 vs 2.9±1.0; $p>0.05$). The addition of more one auricular Norgestomet implant did not alter the analyzed variables. The 12h delay in ovulation induction reduced the number of >8 mm follicles at embryo recovery ($p<0.05$). Although a statistically significant increase was not verified, the use of two implants (1.5±0.5 vs 3.0±1.0) and the 12 hours delay in ovulation induction (1.6 ± 0.6 versus 2.9 ± 1.0) had improved the number of embryos suitable for freezing in superovulated Holsteins cows. (Acknowledgments: **Intervet**)

SUPERSTIMULATION WITH FIXED-TIME ARTIFICIAL INSEMINATION IN *Bos indicus* DONORS TREATED WITH FSHp OR WITH eCG

Chiari, J.R.[1]; Demétrio, D.[1]; Sá Filho, M.F.[2]; Ayres, H.[2]; Baruselli, P.S.[2]

[1]SAMVET, Morrinhos-GO, [2]Dep.Reprodução Animal, FMVZ- USP, São Paulo-SP, Brasil.
samvetembrioes@vne.com.br; barusell@usp.br

The aim of this study was to evaluate the use of porcine recombinant follicle-stimulating hormone (pFSH) or equine chorionic gonadotropin (eCG) in superstimulation with fixed-time artificial insemination (SOFT) protocols in Guzera donors *(Bos indicus)*. A total of 8 females was assigned in two groups (FSH and eCG). Each female received both treatments (FSH or eCG; Cross-over) with 60 days of interval. The same batch of semen was used per donor in both treatments. In the beginning of treatment (Day 0), all females received an auricular Norgestomet implant (Crestar, Intervet, Brazil) and 2mg of estradiol benzoate plus 50mg of progesterone i.m.(Index Farmacêutica, Brasil). The FSH Group (n=8) was treated with 133mg of FSHp (Folltropin, Bioniche, Canada) administered in 8 decreasing doses at 12h of interval from Day 4. At this same day, the eCG Group (n=8) received a single dose of 2000IU of eCG (Folligon, Intervet, Brazil). On Day 6, two doses (morning and afternoon) of PGF2α (Preloban, Intervet, Brazil) were administered in FSH Group, while the eCG Group received the second dose on Day 7 at morning. The implant withdrawal occurred 24h (P24) and 250mg of GnRH (Fertagil, Intervet, Brazil) were administered 48h after the first PGF2α dose. The response to treatments was evaluated by ovarian ultrasonography. Data were analyzed by Anova test. The results of FSH vs. eCG were, respectively: follicles >8mm at embryo collection (1.1±0.5 vs. 1.4±0.5; p>0.05), *corpus luteum* at embryo collection (6.9±1.2 vs. 7.0±1.2; p>0.05), total of structures (5.9±1.3 vs. 4.5±1.0; p>0.05), transferable embryos (4.4±1.3 vs. 2.9±1.0; p>0.05), embryos suitable for freezing (3.7±1.0 vs. 2.4±0.9; p>0.05), unfertilized structures (1.0±0.9 vs. 1.0±0.6; p>0.05) and degenerated structures (0.5±0.3 vs. 0.6±0.3; p>0.05). Results are suggestive that even with the numeric reduction in the amount of viable embryos, the eCG treatment reduces the number of injections (8 for FSH vs. 1 for eCG) in SOFT protocols.

(Acknowledgements: Intervet)

EFFECT OF SUPEROVULATED REPEATEDLY IN PARDO SUIZO CAWS UNDER TROPICAL CONDITIONAL

Solano, R.F.[1]; Dias, J.M.B.[1]; Solano, O.G.[2]

[1]Universidade Estadual do Maranhão, Laboratório de Genética e Biotecnologia, [2]Aluno de Iniciação Científica.

The mean objective of this experiment was to evaluate the efficiency of two superovulate repeatedly, under ovarian response in Pardo Swiss caws used in embryos transfer programs. This experimental work was done in Caxias Maranhão. The animals had been kept in system of pasture supplemented with grains ration and mineral. The experiment was started in September/1997 and ended in february/1999. In this period were superovulated 17 donors, divided GI (n=9) and GII (n=8) first and second superovulation with at 60 days intervals with gonadotrophin 300UI of Pluset, (Serono Ltda-Italia) between days 9 a 12 of estrus cycle, with intervals of 12 hours. 0n third day, the animals received a luteolitic dose of prostaglandin F2α analogous and inseminated 12 a 24 hours after the mounting activity. In the day of the flushing the ovaries had been appraised by ultrasonography. The structures were evaluated by morphologic criteria according the IETS manual. The medium numbers and standard deviation of the evaluated parameters in two analyzed group were respectively: 6.44±1.21; 7.73±1.44 corpora luteal; 4.78±0.95; 4.22±0.81 collections embryos and 3.17±0.78; 3.11±0.74 viable embryos respectively. In 44.4% (4/9) and 50.0% (4/8) donors were more tree normal embryos in both superovulated repeatedly. The follicles medium > 10 mm observed of days collection was 4.22±1.16 e 3.61±0.97 in GI and GII respectively. In conclusion, in spite os number of animals reduced, the repeat superovulation in intervals at 30 and 60 days non had effected in superovulation repeat in Pardo Swiss caws.

SUPERESTIMULATION IN GENOTIPIC CAWS MAMBI DE CUBA (3/4H x 1/4Z) INDUCED WITH FSH-P AND PMSG IN TROPICAL CLIMATE CONDITION

Solano, R.F.[1]; D'Oliveira-Sousa, A.[1]; Carballo, J.I.[2]; Fuentes, D.S.[2]; Veliz, J.[2]; Solano, O.G.[3]

[1] Universidade Estadual do Maranhão, [2] Universidade Estadual Havana Cuba, [3] Acadêmico bolsista de Iniciação Científica, solanoferro@yahoo.com.br

Previous studies show that cows raised on tropical climate are more responsive to gonadotrophyns than cows raised under temperate climate. The aim this study was to evaluate the embryo production of Mambi of Cuba cows superovulated with different treatments of gonadotrophin The animals were devide in GI (n=15) received 2,000 UI of PMSG and 48h after PGF2α and GII (n=25) treatments with 220mg of FSH-P (Folltropin, Vetrepharm, Canada) divided into eight decreasing dosis 35mg Bid; 30mg Bid; 25mg Bid; 20mg Bid). The donnors received 500 μg of PGF2α. (Estrumate, ICI-80996) with the fifth injection of FSH, and were inseminated 12 and 24 hours after the initiation of estrus. The embryos were collected by cervical route on day 7 and 8 after the insemination and the recovered embryos were evaluated by morphology criteria according the IETS manual. The observation of the ovaries was done by laparoscopic, were analyzed the number of corpora luteal (CL); collected structures (CE) and embryos viability (EV). The results were analyzed by student Test. In average, there were significative difference ($P<0.05$) between the CL and EV (8.30±1,15 and 5.50± 0.85) for GI, in comparison with GII (5.20±1.64 e 2.13± 2.12 respectibily).There was tendency ($P<0.06$) for number the follicles persistent 5 to 8 mm (2.83±0.15 vs 4.52±0.82 for GI and GII, respectibily). In 13.3 % (2/15) showed degenerations follicles and non fertilized structures. These results showed that superestumulation with FSH-P are more efficient than PMSG in Mambi de Cuba cows.

ADMINISTRATION OF DIETS WITH SEED OF CANOLA AND SEED OF LINSEED IN THE SUPEROVULATORY RESPONSE, PRODUCTION AND QUALITY OF EMBRYOS IN NELORE COWS *

Albuquerque, K.P.[1]; Capovilla, L.C.T.[2]; Prado, I.N.[3**]; Cavalieri, F.L.B.[2]; Rigolon, L.P.R.[2]; Ramos, F.S.[4]; Tesolin, L.C.[5]

[1]Doutoranda de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá - UEM Av: Colombo, 5790 - 87020-900 Maringá-PR. [2]Professor Doutor do Centro Universitário de Maringá - CESUMAR Av: Guedner, 1610, 87050-390 Maringá-PR. [3]Professor Doutor Titular do Departamento de Zootecnia da Universidade Estadual de Maringá - UEM Av: Colombo, 5790 - 87020-900 Maringá-PR. **Pesquisador Bolsista de Produtividade CNPq. [4]Médica Veterinária do Centro Universitário de Maringá - CESUMAR Av: Guedner, 1610 - 87050-390 Maringá-PR. [5]Aluna de Graduação em Zootecnia da Universidade Estadual de Maringá - UEM Av: Colombo, 5790 - 87020-900 Maringá-PR. lcapovilla@wnet.com.br * Dados preliminares de tese de doutorado em andamento no Programa de Pós-Graduação em Zootecnia (PPZ) da Universidade Estadual de Maringá (UEM).

The suplementation with lipid has been studied by causing beneficial effects in the reproduction, as the increase in the sanguine concentration of progesterona, size of the ovulatory follicle, number of ovarian follicles, modulation of the corporea lutea, conception and gestation rate (Staples et al., 1996). The objective was to evaluate the effect of the sources lipids in ômega-9 (canola seed - CAN) and ômega-3 (linseed seed - LIN) in comparation with lipid absence (SLI) about the answer superovulation, production and quality of embryos in Nelore cows. The experiment was accomplished in Experimental Center of Iguatemi-UEM. 17 Nelore cows (P.O.), housed in individual stalls of 10m^2. The feeding period was 46 days that correspond 30 days before the protocol until the flushing of the embryos. The cows were distributed aleatoricment in one of the treatments that were: SLI (n=6), CAN (n=6) and LIN (n=5). The diets were formulated according to NRC (1996), being isoproteics and isoenergetic. The total ration of the treatment SLI was: cotton peel, corn, cotton crumb, limestone and mineral salt 90. The treatment CAN was: of cotton peel, canola seed, corn, limestone and mineral salt 90. The treatment LIN was: cotton peel, cotton crumb, linseed seed, corn, limestone and mineral salt 90. The cows were synchronized with implant, containing 3,0 mg of norgestomet (D0) and shot of 2,5 mg of E.V. in D1 early. In D5, it began the superovulation for four days, being the total of 250 U.I. of FSH. In D7 2 ml of PGF2α was applied early. In D8 the implant was removed and 12 hours after applied hCG. In D10 the cows were inseminated in the period of the afternoon and morning of the following day. The superovulatory response was obtained through the rectal palpation. The flushing was accomplished through routine practice. The evaluation of the embryos as for the development stage and quality was made according to the classification proposed by the manual of IETS - International Embryo Transfer Society (1999): degree I (excellent or good), degree II (to regulate), degree III (poor) and degree IV (dead or degenerate). In the three treatments presence of corporea lutea was observed, being, 83% for SLI, 83% for CAN and 100% for LIN. The recovered structures, for the treatment SLI, was 6,4 total structures, 5,4 transferable structures, there were not degenerate structures and 1,0 structures not fertilized. For the treatment CAN, 11,5 total structures, 7,5 transferable structures, 2,3 degenerate structures and 1,8 structures not fertilized. For the treatment LIN, 8,0 total structures, there were not transferable structures, 2,8 degenerate structures and 5,2 structures not fertilized. Regarding quality of the embryos, for the treatment SLI 52% of occurrence of embryos degree I, 33% embryos degree II and 15% of embryos degree III. For the treatment CAN, 64% of embryos degree I, 18% of embryos degree II and 18% of embryos degree III.

SUPEROVULATORY RESPONSE IN NELORE HEIFERS (*Bos indicus*) TREATED WITH 250 OR 500 UI OF FSHp

Santiago, L.L.[1]; Torres, C.A.A.[2]; Nogueira, E.T.[3]; Amorin, L.[2]; Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo/ SP;
[2]Departamento de Zootecnia, UFV – Viçosa/ MG; [3] Rua Joaquim Távora, 541, São Paulo/SP, 04015-001, Brasil. s21luciene@hotmail.com e barusell@usp.br

The aim of this study was evaluate superovulatory response in animals treated with 250 or 500 UI of FSHp (Pluset®). It was tested the hypotheses that animals superovulated with higher dosage would present lower superovulatory response due to the fact that FSHp has 50% FSH:50% LH. Twenty seven Nelore heifers were randomly allocated to the following groups: 250 (n=13) or 500 UI of FSHp (n=14). The treatment started nine days after estrus detection (d=0), in 8 injections with decreasing doses, receiving a total of 10 mL. At time of the sixth and seventh doses all cows were treated with PGF2α. Artificial insemination was performed at 12, 20 and 28 after the onset of oestrus. Statistical assays were accomplished using SAS Institute Inc, Cary, NC, USA. Differences between averages were calculated by *t*-Student Test. All heifers treated with 250 UI of FSHp ovulated compared to only 64% of heifers treated with 500 UI of FSHp. In addition, low dose tended to present greater embryos/ova recovered per flush (8. 9 ± 1. 6 vs 4. 6 ± 1. 8, P= 0.10). The number of degenerate embryos per flush tended (P= 0.09) to be greater for 250 UI of FSHp (3.4 ± 0.8 vs 1.5 ± 0.7) and this dosage presented greater (P= 0.03) unfertilized embryos per flush (1.0 ± 0.4 vs 0.1 ± 0.1). There was no difference between groups for fertilized embryos (7.7 ± 1.5 vs 4.4 ± 1.8, P= 0.20), viable embryo (4.5 ± 0.9 vs 3.0 ± 1, P= 0.37) and frozen embryo (3.7 ± 0.8 vs 2.1 ± 0.9, P= 0.22). In conclusion, the results of this experiment confirm the data of previous one whose indicate the treatment with 500 UI of FSHp may be take to previous luteinization of the follicles during superovulation in reason of have high amount of LH (SANTIAGO et al., IRAC -2005). However, studies using more animals by treatment could be valuable to corroborate the data of this experiment.

OOCYTE TRANSFER INTO BOVINE OVIDUCT OF FEMALES WITH SINGLE AND MULTIPLE OVULATIONS

Carvalho, N.A.T.[1,2]; Gimenes, L.U.[1]; Sá Filho, M.F.[1]; Vannucci, F.S.[1]; Reichert, R.H.[2]; Carvalho, J.B.P.[3]; Andrade, J.B.[4]; Pires, R.M.L.[4]; Alvarez, R.H.[4]; Meirelles, F.V.[5]; Chiari, J.R.[6]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ-USP-SP, 05508-000; [2]APTA-Vale do Ribeira; [3]APTA-Pindamonhangaba; [4]IZ-Nova Odessa; [5]FZEA-USP; [6]SAMVET, Morrinhos-GO.
nelcio@usp.br

The aim of the present study was to evaluate bovine and buffalo oocyte transport through genital system of bovine females with single and multiple ovulations. Eight bovine females were assigned in four groups (2x2 factorial). All females received 2mg of estradiol benzoate i.m. (Estrogin®, Farmavet, Brasil) and a progesterone intravaginal releasing device (DIB®, Syntex, Argentina) on random day of estrous cycle (Day 0). On Day 4, the animals of G2 and G4 groups (multiple ovulations), received 2500UI of eCG i.m. (Novormon®, Syntex, Argentina). On day 8, DIB® was removed associated to 0.150mg of PGF2α i.m. (Prolise®) administration in all animals, and also for that females of G1 and G3 groups (single ovulation) 400UI de eCG (Novormon®) was given. On Day 9, GnRH i.m. (Gestran Plus®; 50μg de Lecirelina, Arsa, Argentina) was injected and, after 12 and 24 h, the females were inseminated with bovine (G1 and G2) and buffalo (G3 e G4) semen. On Day 10, all females were submitted to a right flank laparotomy and to exposure of oviduct ipsilateral to ovulation (G1 e G3) or ipsilateral to side that had more ovulations (G2 e G4). In G1 and G2 groups, 10 bovine oocytes submitted to maturation were inserted in the infundibulum while in G3 and G4 groups the same process was performed with 10 buffalo oocytes. The recovery of embryo structures was performed by transcervical uterine flushing on Day 15 for G3 and G4 and on Day 16 for G1 and G2 groups, using 1L of DPBS (Cultilab®). The ovulatory rates to G1, G2, G3 e G4 were, respectively: 100.0±0.0%, 89.2±2.5%, 100.0±0.0%, 93.7±6.2%. Eight embryos were recovered in G2 (23.5%; 8/34 – transferred oocytes + ovulations) and only one in G3 (9.1%; 1/11). No structures were recovered in G1 and G4 groups. The insertion of oocytes in oviduct produced edema in this structure, probably compromising the gametes transport, leading to low embryo recovery rate.

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STATE OF DEVELOPMENT AND PREGNANCY RATE OF DEMI-EMBRYOS

Fernandes, C.A.C.[1]; Oba, E.[2]; Figueiredo, A.C.S.[3]; Ludgero, B.F.A.[3]; Oliveira, E.R.[3]; Gioso, M.M.[4]

[1]Bolsista FAPESP, Pós-Doutorando Unesp-Botucatu-18618-000, Brazil; [2]Profa. RARV-Unesp-Botucatu; [3]Biotran Ltda; [4]Doutoranda Unesp-Botucatu.

One of the alternatives to increase the pregnancy rate in the embryo transfer programs would be the embryo split. Embryos of good and excellent quality could be bipartite originating two hemi-embryos of which the added pregnancy rate would be superior to the intact embryo that originated them (Khurana & Niemann, 2000). Beyond the quality, the development phase of the original embryo can modify the final pregnancy rate. The objective of this study was to verify the pregnancy rate of hemi-embryos originated of intact structures at different development phases. It was been split embryos in the period morulae to blastocysts with morphologic evaluation of excellent (degree 1). The embryos, bipartite or not, had been innovulated by the same technique. They had been distributed for the recipients of the following. Group 1 (Control - n=50) intact embryo. Group 2 (hemi-morulae: n=18), group 3 (hemi-early blastocysts: n=19), group 4 (hemi-blastocysts: n=22). It was been compared by the χ^2 between the groups the average pregnancy rate to the 28-31 days evaluated by ultrasonography. Related to the total of original embryos (intact) used by group the pregnancy rate was 55.77^{a}; 62.50^{a}; 87.50^{b} and $109.09\%^{c}$ for groups 1, 2, 3 and 4 respectively ($P<0.05$). Although the embryos split is easy in the period of morulae therefore is not depend of the orientation, the lesser cell number in embryos in this phase favors the biggest loss (Escriba et al., 2002). Blastocysts for presenting cells in bigger number and smaller probably showed minor loss of stability and cohesion of the cellular mass, therefore the best pregnancy rate in hemi-embryos originated from others of more advanced development phases. Similar results had been obtained in this study where split early blastocysts and blastocysts had presented better pregnancy rates.

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PREGNANCY CHARACTERISTICS DERIVED FROM BOVINE RECONSTITUTED *IN VITRO* HALF EMBRYOS

Alvarez, R.H.[2]; Amaral, J.B.[2]; Pires, R.M.L.[2]; Martinez, A.C.[2]; Oba, E.[3]

[2] Centro de P&D Genética e Reprodução Animal, Instituto de Zootecnia-APTA, Nova Odessa-SP, 13460-000, Brasil. [3]Depto de Reprodução Animal e Radiologia Veterinária FMVZ-UNESP, Botucatu-SP, Brasil. herrera@iz.sp.gov.br

There is evidence in literature showing that embryo's manipulation and *in vitro* culture of bovine embryos affect the pregnancy rate, size and weight of the newborn. The present study aimed to evaluate some pregnancy characteristics resulting from transferred bovine hemi embryos reconstituted *in vitro*. Embryos (morulae GI) recovered from superovulated Nelore and Caracu cows were distributed into three experimental groups. Group 1 was constituted by intact embryos transferred into cows and heifers recipients immediately after recovery (n = 36). Group 2 by hemi embryos transferred (a half in each recipient) after bipartition with a micro blade (n=38) and group 3 by hemi embryos transferred after culture (in Earle medium + 10% SFB at 38,5 ^{o}C and 5% CO_2) during 24 hours (n=34). Data were analyzed by Anova and Chi square test. The development rate to the blastocist stage of *in vitro* cultured hemi embryos was 78.6 %. The pregnancy rate at 45 days was 52.8%, 47.4% and 52.9% for groups 1, 2 and 3, respectively (P>0.05). The calving rate was 30.6%, (group 1), 26.3% (group 2) and 29.4% (group 3) (P>0.05). The average weight (± sem) of the calves at birth was 38.0 ± 4.2 kg (group 1), 32.4 ± 2.5 kg (group 2) and 36.2 ± 2.6 kg (group 3) (P> 0.05). The results suggest that spliced embryos with or without *in vitro* culture may not affect the pregnancy characteristics or the weight of the calves.

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GESTATION RATE OF INTACT OR SPLIT BOVINE EMBRYOS

Fernandes, C.A.C.[1]; Oba, E.[2]; Figueiredo, A.C.S.[3]; Gioso, M.M.[4]

[1]Bolsista FAPESP, Pós-Doutorando Unesp-Botucatu-18618-000, Brazil; [2]Profa. RARV-Unesp-Botucatu; [3]Biotran Ltda; [4]Doutoranda Unesp-Botucatu.

Embryos of good and excellent quality could be split originating two hemi-embryos, of which the added gestation rate would be superior to the intact embryo that originated them (Khurana & Niemann, 2000). An alternative for the reduction of the necessary recipient number in the case of the bipartition would be the inovulation of the two hemi-embryos in one same recipient (Lopes et al. 2001). The aim of this work was to verify the gestation rate of intact and hemi-embryos in a commercial embryo transfer program in bovine. It was been split only embryos in the state of morulae to blastocyst with morphologic evaluation of excellent (grade 1). The embryos, bipartite or not, was been transferred by the same technique. It was been distributed for the recipients. Group 1 (Control - n=50) intact embryo. Group 2 (1 hemi-embryo - n=50). Group 3 (2 hemi-embryos - n=50) two hemi-embryos proceeding from a bipartition. These two hemi-embryos will be transferred together. It was been compared by χ^2 between the groups, the gestation rates to 28-31 and 60-80 days, evaluated for ultrasonography. Related to the total of original embryos used by group the gestation rate to the 28-31 days it was of 55.77^a; 88.88^b and 60.78^a% for groups 1, 2 and 3 respectively ($P<0.05$). At 60-80 days the gestation rate was 51.92^a; 70.37^a and 52.94^a% for groups 1, 2 and 3 respectively ($P>0.05$). The biggest embryonic loss between the two periods of evaluation of the gestation in group 2 made that the significant difference of this variable between the groups observed to the first period did not exist to the 60-80 days. The results show that the gestation rate of hemi-embryos must more delayed be evaluated, therefore the incidence of embryonic loss are bigger.

KHURANA, N.K. & NIEMANN,H. **Theriogenology**, 54; 2, 313-326, 2000.
LOPES, et al. **Theriogenology**, 58; 1383-1392, 2001.

EMBRYO BIPARTITION EFFECTIVENESS OF NELORE BREED CONSIDERING PREGANCY RATE.

Silveira, E.C.[1]; Constantino-Max, M.[2]; Blaschi, W.[3]; Padilha, L.C.[3]; Francisco, R.C.[1]; Pansard, H.S.[1]; Bortolotti, L.A.[1]; Sgobero Filho, I.[1]; Seneda, M.M.[3]

[1] Produtiva – Centro de tecnologia pecuária, Londrina –Pr, 86060-450, Brasil. [2] Médica Veterinária Autônoma, Londrina-Pr, [3] Universidade Estadual de Londrina, Londrina-PR, 86051-990, Brasil. produtiva@zipmail.com.br

In order to increase in vivo embryo production, we analyzed the relation between pregnancy rates of whole embryo and rates pregnancy of Nelore bipartited embryos. Using the invert microscope (Nikon®, TMS) and the micromanipulator (Narishige®, MN 151), we could bipartite morulas and blastocyste classified as great quality. The medium used as bipartition was the Splitting - plus (AB-Nutricell®), and as culture medium was the Holding - Plus (AB-Nutricell®). In the bipartition process we used 39 embryos in vivo. We could obtain 78 hemi-embryos that were transferred immediately to previously prepared receptors cows. In order to be possible a comparison, 125 whole embryos were obtained and transfer under the same condition. For the pregnancy diagnostic, everyone the receptors cows was submited a ultrasonography avaliation after 60 days at embryos transfer. Receptors cows treated with ET using hemi-embryos, pregnancy rate was 53.2 %(42/78) and pregnancy rate for whole embryos transferred was 61.6% (77/125). At the end of experiment there were 42 pregnancies from the 39 embryos subject to bipartition what results in a utilization rate of embryo/pregnancies of 107,7%. Results from this study showed embryo bipartition of Nelore breed as a viable alternative to increase embryo production, particularly, on situation which in vitro production is not able to be done and/or on a situation which donors cows have a good capacity of in vivo embryo production.

BISECTION OF GOAT EMBRYOS – OFFSPRINGS PRELIMINARIES

Rêgo, T.P.[1]; Souza, J.A.[2]; Melo, M.V.L.C.C.[3]; Carmo, D.R.[3]; Sobrinho, J.M.F.[1]; Santos, P. A. C.[1];

Pós-graduanda em Ciência Animal/UFPI[1]; Departamento de Clínica Veterinária/UFPI[2]
adalmir@ufpi.br ; Médicos Veterinários Autônomos[3]

Several options in embryo micromanipulation research are able: transfer of genes, embryo sexing, nucleo-citoplasma relation study, blastomer micromanipulation and embryo bisection, this more viable technique (BEM et al., 1987). The bisection consist in mechanical embryo division, while is possible the obtention of genetically identical product, called monozygotic twins or true identical twins. In livestock species, the first works were performed on ovine (WILLADSEN, 1979) and bovine (WILLADSEN and POLGE, 1981). In Brazil, works in bovine (BEM, 1987), goat (SALLES, 2001) and horses (SILVEIRA, 2004) were performed. In commercial farm, the bisection is an important perspective in embryos transfer programs because result in a gestation number increase. The arm of this work, was evaluate the bisection technique viability in goats. Was utilized ten Boer donors synchronized with progesterone (CIDR, Pharmacia, Brazil) during 11 days and superovulated with 500IU of FSH/LH (Pluset, Callier) in six injections at 12 hours interval beginning 3 days before progestagen removal. Recipients synchronized progestagen (Sponge-Syntex-Argentina), receive 300IU of eCG (Folligon, Intervet, Brazil) and 75 µg of Cloprostenol (Ciosin-Coopers, Brazil). The collection of embryos was made by cervical via seven days after first oestrus detection, with pinces, speculum and nasogastric sundae. Of 80 structures recouped, 39 (morulae or blastocist) of quality I and II were used. Of three embryos gotten one was biparted and the halves inoculates in one same receiving, while entire embryos had been inoculates in pair. For bisection, a composed micromanipulator for two arms supported in a suitable base to a stereomicroscopic was used. The embryo was fixed by a suction micropipette, kept in one of the arms and located, to carry through the cut. The hemi-embryos were planted and inovulated by semi-laparoscopy. The gestation diagnosis was carried through transabdominal ultrasound 30 days after the inovulation. Of the 13 receiving inovulated with entire embryos, seven (53.84%) presented perches, four with two embryos (having corresponded 42.31% of the transferred embryos). Of the 13 that received hemi-embryos, two (15.38%) presented perches of an embryo and one (7.69%) of two embryos, totalizing 30.76% of the transferred embryos. Parted embryos in morulae stage perriod, independent of quality degrees, had no gestation, while blastocist stage period the index was 66.6% to structures of quality I. It is concludes that the morulae stage period is not indicated for bisection of embryos in goat, when gestation increasing is the objective in an rigorous embryos classification is necessary to best results.

FERTILITY OF RECIPIENTS AND EMBRYO SURVIVAL AFTER TRANSFER OF MORADA NOVA (VARIETY WHITE) OVINE EMBRYOS SUBMITTED TO TWO PROCEDURES OF THAWING

Lopes Júnior, E.S.[1]; Maia, E.L.M.M.[1]; Teixeira, D.I.A.[1]; Padilha, R.T.[1]; Almeida, A.P.[1]; Lima, I.M.T.[1]; Albuquerque, C.P.[1]; Pinheiro, E.S.P.[1]; Rondina, D.[1]; Villaroel, A.B.S.[2]; Freitas, V.J.F.[1]

[1]Laboratory of Physiology and Control of Reproduction - FAVET/UECE, 60740-000, Fortaleza-CE, Brazil. [2] Center of Agriculture Sciences - FZ/UFC, 60455-760, Fortaleza-CE, Brazil. vjff@uece.br

Unlike of ethylene glycol, the sucrose used in thawing procedure of bovine embryos requires a simple protocol that reduces the time of this procedure (Niemann H., Theriogenology, v. 17, p. 102). However, few systematic studies have been performed to determine the best protocol to be used at thawing procedure of ovine embryos. Thus, the aim of this study was to evaluate the embryo survival after transfer of embryos of Morada Nova [variety white (MNw)] ewes cryopreserved in ethylene glycol (ETG) and thawed in ETG or sucrose (SUC). For this, embryos were submitted to low freezing (Baril G., Manual de Formación Práctica para el Trasplante de Embriones en Ovejas y Cabras, 182p), using 1.5 M ethylene glycol in a programmable freezer (Dominium K, BIOCOM, Brazil). The estrus of fourteen crossbred ewes was synchronized with a vaginal sponge containing 60 mg of medroxyprogesterone acetate (Progespon®, Syntex, Buenos Aires, Argentine) for 14 days. At sponge removal, recipients received 300 IU eCG (IM). Just prior embryo transfer, which was performed six days after estrus onset, embryos were thawed by immersion in water bath at 37°C during 30 s and, after this, they were transferred to solutions containing ETG (n = 17) or SUC (n = 13). Embryos in ETG group were plunged in decreasing concentrations of ETG (1.5 M, 1.0 M, 0.5 M and 0.0 M) each five minutes, while the embryos in SUC group were plunged in two solutions of 0.25 M followed by more two of 0.0 M of SUC, each five minutes too. After this, recipients were submitted to laparoscopy in order to evaluate the ovarian response, followed by embryo transfer, which was performed by semi-laparoscopy. Each recipient with, at least, one functional corpus luteum, received two or three embryos at ETG (n = 7) and SUC (n = 7) groups. Pregnancy was diagnosed 30 days after embryo transfer by transrectal ultrasonography (240 Parus, Pie Medical, Holand) using a real-time B-mode scanner equipped with a 6-8 MHz transducer. Data were expressed as mean ± SEM or percentage. Embryo survival and fertility rates were compared by Chi-square test. It was observed 100.0% of recipients in estrus. The mean interval sponge removal to estrus onset was 33.7 ± 5.8 h, while ovulation rate was 2.0 ± 0.3. ETG group showed a higher embryo survival (ETG = 55.6% vs. SAC = 0.0%; $P<0.05$) and a higher fertility rate (ETG = 57.1% vs. SAC = 0.0%; $P<0.05$). Data permit conclude that thawing procedure using ETG show to be more efficient to induce better fertility and embryo survival rates in MOET programs that use frozen-thawed embryos of MNw ewes.

SERUM CONCENTRATIONS OF PROGESTERONE IN OVARIECTOMIZED EWES TREATED WITH LONG ACTING PROGESTERONE (P4LA-150)

Almeida, A.K.[1]; Martins, L.E.P.[1]; Biscarde, C.E.B.[1]; Neves, T.A.[1]; Chalhoub, M.[1]; Ribeiro Filho, A. de L.[1]; Bringel, B.A.[2]; Douglas, R.H.[2] Gusmão, A.L[1].

[1]Escola de Medicina Veterinária/UFBA, Salvador-BA, 40170-110, Brasil. [2] Bet Labs, Lexington - Kentucky, 40511-1209, USA. gusmao@ufba.br; rhdouglas1222@aol.com

Estrus synchronization protocols in ewes predominantly use implants, intravaginal devices or sponges impregnated with progesterone. Both long term treatments of 12 to 14 days and short term 5 to 6 day treatments are used. Reduction of labor, elimination of the risk losing vaginal devices and a reduced incidence of vaginitis can be achieved by using and injectable long acting progesterone. Therefore, the objective of this study was to asses the effect of long acting progesterone (Biorelease P4LA-150, BioRelease Technologies on serum concentrations of progesterone in ovariectomized ewes. Four ovariectomized ewes were used, two of which were of the Santa Inês breed and two Santa Inês x Dorper breed kept in an intensive system. Ewes were randomly assigned to one of four treatment groups in a LATIN SQUARE design so that each ewe was injected intramuscularly with the following doses of Biorelease P4LA-150: 0, 75, 150 or 300 mg. Blood samples of all animals were collected before the progesterone administration and on D1 (day 1); D2; D5; D7; D8; D9; D10; D11 and D12 after the dose. The analysis of serum progesterone was performed by radioimmunoassay in solid phase (DPC-Medlab) and the data were evaluated analysis of variance. The results show that the concentrations of 75 and 150 mg kept the serum progesterone levels over 1 ng/mL until D5 and the dose of 300 mg until D9. In conclusion P4LA-150 can be used to keep the levels of progesterone over 1 ng/mL until D5 or D9. Studies with cyclic animals should be done to study the efficacy of the product and of the tested doses in estrus synchronization protocols in sheep.

SUPEROVULATORY RESPONSE IN LACTATING SAANEN DOES USING SHORT-TERM PROGESTERONE PROTOCOL AND RECOMBINANT BOVINE SOMATOTROPIN (RBST)*

Fonseca, J.F.[1]; Viana, J.H.M.[2]; Bruschi, J.H.[2]; Zambrini, F.N.[2]; Palhão, M.P.[2]; Santos, A.F.A.[2]

[1]Embrapa Caprinos, Estrada Sobral/Groaíras, Km 4, CP D10, 62.011-000, Sobral-CE, Brasil, jeferson@cnpc.embrapa.br [2]Embrapa Gado de Leite, Rodovia MG 133, Km 42, 36.155-000, Cel Pacheco-MG, Brasil.

The objective of this study was to investigate the efficiency of two short protocols on superovulatory response in goats. Five lactating Saanen does were tested in a cross-over design for two treatments (T1 and T2). In both treatments, intravaginal progesterone devices (CIDR®) were inserted (day 0) and removed six days later (day 6) and 22.5 μg cloprostenol were administered by intravulvo-submucosal via (day 0). On day 4, does started to receive six decreasing doses of FSH (250 to 400 IU) with 12 hours interval. Three 50 mg flunixin meglumine doses were administered on days 9, 10 and 11. In T1 and T2, animals received two doses of saline or 250 mg of rbST on day 0 and first estrous detection, respectively. Does were mated until the end of estrus and uterus were transcervically flushed seven days after first mating. Interval between superovulations was superior to 60 days. One doe did not respond to T2. One doe presented 27 (19 oocytes) and 26 (22 oocytes) structures for T1 and T2, respectively. Total structures (18.4 ± 9.4 and 15.7 ± 11.0) and percentages of oocytes (29.3 and 34.9), viable (63.0 and 52.4), grade-1 (29.3 and 30.2), grade-2 (20.7 and 6.3), grade-3-4 (13.0 and 15.9) and degenerated embryos (7.6 and 12.7) did not differ (P>0.05) between T1 and T2, respectively. Short-term progesterone protocol was efficient for superovulation in lactating Saanen does but association with rbST did not enhance embryo recovery or quality.

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PRELIMINARY RESULTS USING A NEW SUPEROVULATORY TREATMENT IN GOATS: THE DAY 0 PROTOCOL

Menchaca, A.[1]; Vilariño, M.[1]; Rubianes, E.[2]

[1]Departamento de Fisiología, Facultad de Veterinaria; [2]Departamento de Producción Animal, Facultad de Agronomía, Montevideo, Uruguay. alejomen@adinet.com.uy

When gonadotropin treatment begins in absence of a dominant follicle, a higher superovulatory response is obtained in goats. The "Day 0 Protocol" was designed to begin the FSH administration soon after ovulation (Day 0) at the moment of emergence of wave 1, which ensures the absence of dominant follicle (Rubianes and Menchaca, Anim. Reprod. Sci. 2003;78:271-287). The present work compares the Day 0 Protocol versus Traditional Protocol in multiparous Alpine goats during anestrous season (October-December, Uruguay, 34° SL). On Day 0 Protocol (n=17) the ovulation was synchronized before FSH administration using a short progesterone treatment (Menchaca and Rubianes, Reprod. Fertil. Develop. 2004; 16:403-414), and FSH administration began soon after ovulation detected by ultrasonography (7.5 MHz, Aloka 500, Japan). Traditional treatment (n=20) was initiated with the insertion of a CIDR-G (0.3 g progesterone, InterAg, New Zealand) for 11 days, and FSH administration began at day 9 associated with one dose of PGF2α analogue (160 μg, delprostenate, Glandinex, Universal Lab, Uruguay). In both groups the superovulatory treatment consisted in six equal doses of FSHp (12 h apart, 200 mg NIH-FSH-P1, Folltropin, Bioniche, Canada). On Day 0 Protocol two half doses of PGF2α analogue (each 80 μg) was given together with the two last FSH doses. In both protocols one dose of GnRH analogue (8.0 μg, buserelin acetate, Receptal, Hoechst, Argentina) was administered 12 h after the last FSH dose and timed artificial insemination using frozen semen was performed 16 and 24 hours later. Follicular diameter of the largest follicle and number of small follicles (<4.0 mm) was determined by transrectal ultrasonography at the onset of FSH treatment. The number of corpora lutea (CLs) was determined by laparoscopy (Karl Storz, Germany) at the day of embryo collection (7 days after insemination). Embryos were collected after laparotomy under inhalatory anaesthesia (Halothane, Rhône-Poulenc, Uruguay). Three goats showed premature regression of CLs (two for Day 0 and one for Traditional protocol, respectively) and were not flushed. Data were compared using ANOVA after log transformation. The diameter of largest follicle at onset of FSH treatment was smaller for Day 0 Protocol (4.1±0.2 mm) than for Traditional Protocol (6.6±0.4 mm; $P<0.001$). In addition, at the moment of the first FSH dose the number of small follicles was higher for Day 0 Protocol (16.7±1.7) than for Traditional Protocol (9.3±1.1; $P<0.001$). At the flushing day, the mean number of CLs and transferable/ collected embryo rate showed a tendency to be higher for Day 0 Protocol (13.8±2.3 and 85/135, respectively) than for Traditional Protocol (10.9±1.9 and 85/161, respectively; $P<0.1$). The results confirm the effectiveness of Day 0 Protocol to start a superovulatory treatment in absence of a large follicle. This preliminary data may suggests successful results for the Day 0 Protocol in number of CLs and transferable embryos compared with Traditional superovulatory treatment in goats.

EMBRYO TRANSFER IN GOATS: INOVULATION BY LAPAROTOMY

Souza, J.A.T.[1]; Melo, M.V.L.C.C.[2]

[1]Departamento do Clínica e Cirurgia Veterinária / UFPI, adalmir@ufpi.br; [2]Fazenda Santo Antônio, Campo Maior-PI;

The embryos transfer (ET) is a handling technique that increases the reproductive capacity of females of high genetic value. In the bovine, that technique is well consolidated, while in the small ruminant, particularly ovine, the trans-cervical access is considered limitant factor. A program of ET consists on the following stages: the donors and receptors selection, estrous syncronization, superovulation, fertilization, collect, evaluation, inovulation and embryos conservation (Andrioli-Pinheiro, 1993). The success of this technique is related to factor associated to donors, embryos and receptors and environmental interaction. In spite of the transcervical access in the goat, inovulation, commonly, it is accomplished by laparotomy, laparoscopy or semi-laparoscopy, this finishes more recommended (Freitas & Simplício, 2002; Brebion et al., 1992), for providing equivalent fertility rates or superior to the laparotomy. The objective of this work was to register the effectiveness of the ET with inovulation accomplished by laparotomy, in goat of the Boer race. The data constitute registrations of 43 collects, accomplished in Santo Antônio Farm, district of Campo Maior–PI. The females were superovulated with 300UI of FSH (Pluset®), in decreasing doses, every 12 hours, starting from the nineth day after application of the progestagen (CIDR®). Implant was retired in the afternoon of the 11 and in the morning and afternoon of that day they were applied 50mg of cloprostenol (Ciosin®). The breeding was made at the first estrous sign and every 12 hours until the ceasing of the symptoms. The collects occurs seven days after the first breeding, trans-cervical via, without tranquilization, after pinched and traction of the cervix with the aid of a human vaginal speculum and two Allis forceps. For uterine wash it was used 180ml of PBS in three moments of 60ml, with the aid of a catheter Russh 12, being the liquid recovered in filter, at the time in that it was introduced in the uterus. After identification and classification of the structures according IETS, the embryos were maintained in PBS medium with 0.4% of BSA to the moment of the inovulation, these done by laparotomy, after receptor tranquilization with xylazine (Rompun®). The ovaries were exposed to evaluate the bodies luteum and the embryos put in the horn corresponding to the bodies luteum of better quality. 43 collects were accomplished with obtaining of 360 structures, being 174 morula, 105 blastocysts, 30 no fertilized and 51 degenerate, corresponding to averages of 8.37±7.18; 4.05±4.05; 2.44±4.28; 0.70±2.02 and 1.19±1.61 structures for collection, respectively. Of the 279 viable embryos (6.49±6.16 for collection), 264 were transferred for 144 receptors: 28 received one, 112 two and 4 three embryos. The gestational rate to the 30-45 days, obtained by ultrassound, independent of the number of received embryos was 56.98%, being 56.10% simple and 43.90% double gestation. In spite of the laparoscopy or semi-laparoscopy techniques are more frequently recommended, our data allow concludes that inovulation by laparotomy is viable.

EARLY PREGNANCY DIAGNOSIS BY REAL-TIME ULTRASONOGRAPHY IN SHEEP RECIPIENTS RECEIVING MORADA NOVA (WHITE VARIETY) EMBRYOS

Santos, M.H.B.[1]; Lopes Júnior, E.S.[2]; Paula, N.R.O.[2]; Arruda, I.J.[2]; Andrade, M.L.L.[2]; Padilha, R.T.[2]; Freitas, V.J.F.[2]; Oliveira, M.A.L.[1]

[1]Program of Post-graduation in Veterinary Science - FAVET/UFRPE, 52171-900, Recife-PE, Brazil [2] Laboratory of Physiology and Control of Reproduction - FAVET/UECE, 60740-000, Fortaleza-CE, Brazil. maico.henrique@uol.com.br

In order to avoid the extinction of Morada Nova (variety white) (MNw) ewes, efforts have been performed in order to preserve the genetic characteristics of this breed. Thus, embryo transfer applied in programs of breed preserving can be an important tool. However, it is essential that early pregnancy diagnosis in embryo recipients is performed in order to use them in a new consecutive program of embryo transfer. This study had as objective to use a real-time ultrasonography to obtain early pregnancy diagnosis in sheep recipients and to observe the migration time of genital tubercle (GT) in MNw breed fetuses. MNw embryos were previously recovered of superovulated donors and they were frozen in a programmable freezer (Dominium K, BIOCOM, Brasil). The estrus of fourteen crossbred ewes was synchronized, using a vaginal sponge containing 60 mg of medroxyprogesterone acetate (Progespon®, Syntex, Buenos Aires, Argentine) for 14 days. At sponge removal, recipients received 300 IU eCG (IM). Six days after estrus onset, embryos were thawed and recipients were submitted to embryo transfer, which was performed by semi-laparoscopy, using two or three embryos for each female. Pregnancy was diagnosed 30 days after embryo transfer by ultrasonography (240 Parus, Pie Medical, Holand) and using a transrectal probe of 6-8 MHz. In addition, fetal-sex determination was performed each 48 h, between 36th and 53rd day of pregnancy. The fetus was recorded as a male when GT was located immediately caudal to the umbilical cord and as a female when it was located near the tail. Data were expressed as mean ± SEM or as percentage. Pregnancy rate was 28.6% (4/14) and 25% of pregnant recipients carried twins. It was observed five fetuses and 60% of them were classified as males. The ultrasonographic definition of fetal-sex carried out 41.0 ± 3.0 days after embryo transfer. In conclusion, real-time ultrasonography showed to be a useful to early pregnancy diagnosis and to verify the migration time of GT in sheep fetuses of MNw breed.

FETAL SEXING BY ULTRASONOGRAPHY IN EWE OF SANTA INÊS BREED

Santos, M.H.B.[1]; Moraes, E.P.B.X.[1]; Rabelo, M.C.[1]; Melo, A.N.[2];
Lima, P.F.[3]; Oliveira, M.A.L.[3]

[1]Programa de Pós-Graduação em Ciência Veterinária/UFRPE; [2]Curso de Graduação em Medicina Veterinária/UFRPE; [3]Departamento de Medicina Veterinária/UFRPE (maloufrpe@uol.com.br)

The present study aimed to identify the sex and the day of genital tubercule (GT) migration of ovine fetuses using real time ultrasonography. In all experiments (EI, EII, EIII), the sex was identified by localization of GT, penis, prepuce, scrotal bag, nipples and genital swelling. In EI, the pregnant uterus (n = 32) between 5 and 10 weeks, obtained in slaughter house, were examined after immersion in a water containing recipient, with 6.0 and 8.0 MHz linear transducer. In EII, the females (n = 17) were monitored with 12 hour intervals from the 35th to the 46th day of pregnancy, by transrectal via with linear transducer (6.0 and 8.0MHz) and by transvaginal via with a micro-convex transducer (5.0 and 7.5MHz). In EIII, the females (n = 30) with pregnancy period from 55 to 75 days were examined once only, using the same transducers and via used in EII. In EI there was an accuracy of 90.6% (29/32) in diagnosis and the mistakes were in fetuses sexed as females. Among 17 females in EII, 11 (64.6%) fetuses were correctly sexed independent of single (7/11), twin (3/11) or triple (1/11) pregnancy In 6 (35.4%) pregnancies, 3 (17.7%) were twin, being impossible to sex one fetus of each pregnancy. In other 3 (17.7%) pregnancies the fetuses were correctly sexed, although the birth did not coincide with the quantification. In a male fetus of a single pregnancy, the migration of the GT began on day 37 of pregnancy and on the 46th day all the fetuses of the other pregnancies were correctly sexed. Among 30 females in EIII, 16 (53.4%) pregnancies were single being sexed with accuracy of 100%. In other 14 (46.6%) remainder the pregnancies were twin, being impossible, in four cases, to be determined the sex of one of each twin. The incorrect diagnoses were fetuses sexed as females, however born as males. From the all born fetuses the total accuracy was 88.9% (EI), 88.0% (EII) and 90.9% (EIII), being not observed difference ($P > 0.05$) amog the experiments. The results allow to concluded that the transvaginal via may help in the identification of the fetal sex and that the ideal period for fetal sexing in Santa Inês breed is from the 50th to the 70th day of pregnancy by transrectal via.

FETAL SEXING IN GOATS BY ULTRASONOGRAPHY

Santos, M.H.B.[1]; Moraes, E.P.B.X.[1]; Moura, R.T.D.[1]; Chiamenti, A.[1]; Bezerra, F.Q.G.[2]; Aguiar Filho, C.R.[2]; Lima, P.F.[3]; Oliveira, M.A.L.[3]

[1]Programa de Pós-Graduação em Ciência Veterinária/UFRPE [2]Curso de Graduação em Medicina Veterinária/UFRPE [3]Departamento de Medicina Veterinária/UFRPE (maloufrpe@uol.com.br)

The aim of this work was to identify the sex and the day of genital tubercule (GT) migration of caprine fetuses using real time ultrasonography. In all experiments (EI, EII, EIII, EIV), conducted in different periods and farms, the sex was identified by localization of GT, penis, prepuce, scrotal bag, nipples and genital swelling. In EI, realized in slaughter house, were used uterus (n = 54) of pregnant females, between 5 and weeks, with out defined breed. After immersion in a water containing recipient the uterus were examined with 6.0 and 8.0 MHz linear transducers. In EII, developed in farm A, the females of Anglo-Nubian breed (n = 33) with pregnancy between 111 and 122 days were submitted to a single exam with micro convex transducers (5.0 and 7.5 MHz) by transabdominal via. In EIII, performed in farm B, the females of American Alpine breed (n = 12) with pregnancy between 45 and 70 days were also examined once with linear transducer (6.0 and 8.0 MHz) by transrectal via. In EIV, conducted in farm C, the females of Anglo-Nubian breed (n = 8) were daily monitored from day 35 to day 60 of pregnancy, with linear transducer (6.0 and 8.0Mhz) by transrectal via and with the micro convex (5.0 e 7.5 MHz) by transvaginal via. Of the quantified fetuses in single pregnancy, 85.7% (EI), 64.3% (EII), 72.0% (EIII) and 100% (EIV) were correctly sexed. Of the quantified fetuses in twin pregnancy, 76.9% (EI), 55.3% (EII), 100% (EIII) and 75.0% (EIV) were correctly sexed, being not possible, in this kind of pregnancy, identify the sex in 23.7% (EII) and 20.0% (EIII) of the fetuses and quantify 25.0% of them (EIV). The migration of GT in a male fetus of Anglo-Nubiana breed took place on the 48th day of pregnancy and on the 55th day all fetuses were correctly sexed. The false diagnosis resulted from fetuses sexed as females and the percentage of the correct diagnosis do not differ between the fetuses sex and among single and twin pregnancies. The results allow to conclude that the transvaginal via may only used to aid the fetal sexing and that the real time ultrasonography is efficient to sex caprine fetus between the 55th to the 70th day of pregnancy.

INDUCTION ESTRUS IN GOATS USING REUSED INTRAVAGINAL DEVICES*

Zambrini, F.N.[1]; Fonseca, J.F.[1]; Bruschi, J.H.[2]; Viana, J.H.M.[2]; Palhão, M.P.[2]; Santos, A.F.A.[2]

[1]Embrapa Caprinos, Estrada Sobral/Groaíras, Km 4, CP D10, 62.011-000, Sobral-CE, Brasil, jeferson@cnpc.embrapa.br. [2]Embrapa Gado de Leite, Rodovia MG 133, Km 42, 36.155-000, Cel Pacheco-MG, Brasil.

This study aims to test the efficiency of induction of estrus in goats during the breeding season. Twenty one goats (08 Toggenburg, 10 Saanen and 03 crossbred) were randomly assigned, according to breed, into three treatments: T1 (n = 08), goats received 22.5 μg cloprostenol intravulvo-submucosal; T2 (n = 7) and T3 (n = 6), goats received for six days intravaginal devices containing progesterone (CIDR®), previously used for 6 and 12 days, respectively plus 22.5 μg cloprostenol at the moment of device insertion and 200 IU hCG 24 h before device removal. In T1, three goats showed estrus between 44 and 56 h and two goats 452 and 692 h after cloprostenol administration; in T2 and T3, all goats showed estrus between 20 and 32 h after device removal. One goat in T2 and one in T3 lost the device. Interval from device removal to onset of estrus (22.0 ± 4.9 h and 22.4 ± 5.4 h) and duration of estrus (34.0 ± 4.6 h and 33.6 ± 5.4 h) did not differ ($P > 0.05$) between T2 and T3, respectively. Interval to estrus and duration of estrus was negatively correlated ($r = -1$, $P < 0.001$). Pregnancy rate (%) was 37.5, 57.2 and 33.4, respectively. Intravaginal devices containing progesterone can be efficiently used until three times in six days synchrony protocols for induction of estrus in goats.

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PROTOCOLS OF THE SINCHRONIZATION OF ESTROUS AND OVULATION IN RECIPIENTS GOATS

Guido, S.I.[1]; Guido, F.C.L.[1]; Nascimento Filho, E.V.[2]; Oliveira, M.A.L.[1]; Lima, P.F.[1]; Andrade, J.C.O.[2]; Rocha, J.D.[1]

[1]Prog. de Pós-Grad. em Ciência Veterinária/UFRPE; Recife-PE, 50630-650, Brasil.
[2]Médico Veterinário Autônomo. sebastiaoguido@uol.com.br

Was evaluated the effectiveness of estrous and ovulatory synchronization protocols in goats, stablished by the new and reused CIDR, intravaginal sponges, eCG and dinoprost. For this purpose were used 60 goats distributed in three groups. In TI (n=20) the animals were treated with a new CIDR for 9 days, being administered on day 7th, via i.m,. 300 I.U. of eCG and 5.0 mg of dinoprost. In TII (n=20) received an vaginal sponge with 50 mg of MAP during 9 days and on day 7th 300 I.U. of eCG and 5,0 mg of dinoprost by i.m. via. The ones of TIII (n=20) were treated, for 9 days, with a CIDR reused, and on day 7th also received 300 I.U. of eCG and 5.0 mg of dinoprost. To determine the plasmatic concentrations of P_4, were randomly chosen 5 females of each group. The blood samples were picked up each 24 hours during the treatment, 24 hours after the withdrawing of the devices and on the 7th day after the beginning of estrous. Also on day 7th after the beginning of estrous, all females were evaluated by laparoscopy to determine the ovulatory rate (TO). All goats submitted to the blood sample collection that ovulated were submitted an ovariectomy for morphological evaluation of CL. The fertility was verified by day 35 using ultrasonography. Were verified estrous percentages of 100% (TI), 80% (TII) e 100% (TIII). The TO average was 2.67 (TI), 2.17 (TII) and 2.33 (TIII). The average concentrations of P_4 during the treatment were 1.76 to 7.74ng/mL in TI and 1.5 to 5.78 ng/mL in TII. On day 7th after the beginning of the estrous were 3.95 ng/mL (TI) and 3.58 ng/mL (TII). The TO after section of the ovaries were 3.75 ± 1.25 (TI) and 3.50 ± 1.50 (TIII), being higher ($p>0,05$) than TII (2,17 ± 1,10). The pregnancy results were 53.3% (TI), 40.0%(TII) e 46.7% (TIII). It may be concluded that the protocols are satisfactory in estrous synchronization and ovulatory rate, as well as that the plasmatic concentrations of P_4 during the use of CIDR are quickly established and continue, even with the used CIDR.

SYNCHRONIZATION OF ESTRUS IN GOATS FOR SHORT DURATION WITH CIDR-G® AND INTRAVAGINAL SPONGE

Maffili, V.V.[1]; Torres, C.A.A.[2]; Fonseca, J.F.[3]; Bruschi, J.H.[4]; Viana, J.H.M.[4]; Prosperi, C.P.[5]; Moraes, E.A.[2]; Pontes, R.A.M.[2]

[1] Departamento de Zootecnia – UFV – vmaffili@cpqgm.fiocruz.br ; [2] Departamento de Zootecnia – UFV; [3] Centro Nacional Pesquisa em Caprinos – EMBRAPA; [4] Centro Nacional de Pesquisa Gado de Leite – EMBRAPA; [5] Departamento de Veterinária – UFLA

The objective of this study was to compare two protocols of short duration synchronization for estrus. Eighteen Toggenburg breed goats have been used, bying twelve multiparous (M) and six nuliparous (N), allocated in two treatments: T1 (n = 6N and 3M) were inserted with intravaginal sponge (ID) impregnated with 60mg of medroxyprogesterone acetate and T2 (n = 6N and 3M) received CIDR-G®. At the insertion time (d=0) 50 µg of PGF was injected in goats from both treatments. On day 5 the ID were removed and the animals were observed for estrous at six hours intervals. Ultrasonographic exams were made daily during the device treatment and at each six hours after the estrus detection, up to 12 hours after the ovulation. Follicular regression occurred at the emergence of a new wave in the day 2.8 ± 0.83 and 3.5 ± 0.53 in the goats from T1 and T2. The estrus and pregnancy rate were of 8/9 and 6/8 for T1 and 8/9 and 5/8 for T2, respectively. The intervals from removal of the device to the onset, and the end of estrus and its duration were: 53.3 ± 14.8 and 36.0 ± 0.0 hours ($P<0.05$), 91.2 ± 13.5 and 71.2 ± 11.8 ($P<0.05$) and 38.3 ± 14.7 and 35.25 ± 11.7 ($P>0.05$) for T1 and T2 goats, respectively. The intervals from the onset and the end of estrus and the removal of the device to ovulation were: 32.2 ± 14.3 and 33.4 ± 7.3 hours ($P>0.05$), -4.3 ± 10.32 and -5.0 ± 7.2 hours ($P>0.05$) and 88.2 ± 16.9 and 69.4 ± 7.3 hours ($P<0.05$) for T1 and T2 goats, respectively. The ovulation number, the diameter and the rate of ovulatory follicle growth were: 2.00 ± 0.63 and 1.8 ± 0.83 ($P>0.05$), 7.3 ± 1.3 and 7.4 ± 1.1 mm ($P>0.05$) and 3.4 ± 1.9 and 3.5 ± 1.2 mm ($P>0.05$) for T1 and T2 goats, respectively. The exposure of goats to different progestagen releasing intravaginal devices showed to be effective, however, it must be taken in consideration the ovulation time in the order to use the different protocols in programs of artificial insemination with fixed time.

EFFICIENCY OF THE CIDR® NEW AND REUTILIZED IN PROGRAMS OF ESTRUS SYNCHRONIZATION OF SHORT DURATION IN TOGGENBURG GOATS

Maffili, V.V.[1]; Torres, C.A.A.[2]; Fonseca, J.F.[3]; Bruschi, J.H.[4]; Viana, J.H.M.[4]; Prosperi, C.P.[5]; Moraes, E.A.[2]; Pontes, R.A.M.[2]

[1] Departamento de Zootecnia – UFV – vmaffili@cpqgm.fiocruz.br ; [2] Departamento de Zootecnia – UFV; [3] Centro Nacional Pesquisa em Caprinos – EMBRAPA; [4] Centro Nacional de Pesquisa Gado de Leite – EMBRAPA; [5] Departamento de Veterinária - UFLA

The objective of this study was to determine the efficiency of the reutilization of the CIDR-G® device for three times. Eighteen Toggenburg breed goats were randomly assigned to three treatments: T1 (n = 6): goats received new CIDR-G®; T2 (n = 6): goats received CIDR-G® used once previously and T3 (n = 6): goats received CIDR-G® used previously for two times. The CIDR-G® was inserted on day zero(d=0) plus 50 µg of PGF analogue (D-cloprostenol) in all the experimental goats. The devices remained for five days in the goats, and when removed, the estrus detection started at four times daily. All the females in estrus were bred naturally. The estrus and pregnancy rates were: 83.3/60% (T1); 83.3/60% (T2) and 100.0/83.3% (T3). The interval CIDR-G® removal to the onset of estrus was shorter for T3 goats (20.0 ± 3.10 hours) than for T1 (37.2 ± 2.70 hours) and T2 ones (33.6 ± 5.40 hours) ($P<0.05$). The estrus length from T3 goats (43.0 ± 8.83) was greater than the ones from T1 (30.0 ± 4.24) ($p<0.05$), but T2 goats (37.2 ± 6.57) ($P>0.05$). There was a negative correlation between estrus length and the interval from CIDR-G® removal to the onset of estrus ($P<0.05$). The device removal to the end of estrus intervals were not affected by the treatments ($P>0.05$). The ovulation number, the intervals from CIDR-G® removal and the onset of estrus to the ovulation time were not affected by the treatments ($P>0.05$). The ovulatory follicle diameter from T3 goats was greater (9.4 ± 0.96 mm; $P<0.05$) than the T1 goats (7.6 ± 1.4mm) and the T2 ones (8.1 ± 0.56mm). But the growth rate of the ovulatory follicle was similar for the animals from the treatments ($P>0.05$). It was concluded that the use of the CIDR-G® for three times was effective to induce estrus synchronization and high gestation rate.

ESTRUS INDUCTION IN TEXELL EWES DURING THE NON-BREEDING SEASON

Castilho, C.[1]; Mori, M.M.[2]; Alessi, C.[2]

[1]Faculdade of Veterinary Medicine, UNOESTE, Campus II, P. Prudente – SP, 19067-175. calie@unoeste.br . [2]Students Faculdade of Veterinary Medicine, UNOESTE, Campus II, P. Prudente – SP

Pregnancy length in ewes is 144 and 151 days, but due to seasonal anestrus caused by photoperiod, ewes only lamb once a year. This constitutes a major limitation to ovine production. Therefore, it would be advantageous to use hormonal protocols that stimulate ovarian activity during the period of anestrus, especially in the Texell breed. Experiments were conducted to evaluate the effect of estrus induction during the non-breeding season in primiparous and multiparous ewes using new or previously used progestagen implants. During 30 days, in September and October, estrous behavior of the used sheep was made (control), with introduction of rams (teaser) per 1 week and after this period had been divided in 2 groups. Group 1 (G-1; n=15) was composed of 12 months old, primiparous ewes that received ½ progestagen auricular implant (Crestar® - Intervet, Holland) and 0.5 mL PGF2α (Day 0) (0.1325 mg cloprostenol, IM; Ciosin® - Schering-Plough Coopers). After nine days the ewes received 2 mL equine chorionic gonadotropin (eCG, 400 IU IM, Folligon® - Intervet, Holland) and progestagen implants were removed 24 hours later (day 10). Group 2 (G-2; n=26) was composed of 36 months old, multiparous ewes, eight weeks postpartum, that received the same treatment described for G-1, except that in this group ewes received previously used progestagen implants (14 implants were used once in cows and 12 implants were used once in ewes. In the day of implants withdraw, two teaser rams fitted with marking harnesses were introduced into the flock and behavior was observed twice a day to determine the onset of estrus. In the G-1, 53.33% (8/15) of the ewes showed estrus approximately 48 hours after implants withdraw. In the G-2, 57.69% (15/26) of the ewes showed estrus, the majority of them (12/15) observed between 48 and 72 hours after implant withdraw. Estrus rate was similar between ewes that received implants previously used in cows (57.14%; 8/14) and ewes that received implants previously used in other ewes (58.33%; 7/12). During the 30 days of estrous behavior (control), made before the beginning of the experiments, without hormone use, no sheep showed estrous manifestation. We conclude that it is possible to induce estrus in over 50% of anestrus, primaparous or multiparous ewes using ½ of a new or previously used progestagen implant and news experiments was conducted.

PROGRAM TO SYNCHRONIZE-INDUCE ESTRUS AND OVULATION FOR 12- TO 14-MONTH-OLD HEIFERS

Bragança, J.F.[1]; Loguercio, R.S.[1]; Escobar, F.R.[1]; Loguercio, L.P.[2]; Oliveira, J.F.[1]; Gonçalves, P.B.D.[1]

[1] Universidade Federal de Santa Maria, Departamento de Clínica de Grandes Animais Laboratório de Biotecnologia e Reprodução Animal - BioRep, Santa Maria, RS, Brasil. bayard@biorep.ufsm.br [2] Granjas 4 Irmãos S&A – Rio Grande – RS – Brasil.

The objective of this study was to develop a hormonal program based in eCG to increase the percentage of follicles which respond to GnRH and to use the corpus luteum as a natural source of gestagen to synchronize-induce estrus in 12- to 14-month-old heifers. Two hundred sixty two Bos taurus (predominant Red Angus) heifers with a body weight of 280 Kg, a body condition score of 4 and 12- to 14-month old, were randomly assigned to three groups: BioRep (n=92), eMGPG (n=85) and eGPG (n=85). In the BioRep group, heifers received a vaginal device containing 250 mg of medroxyprogesterone acetate (MPA) and an injection of 2.5 mg of EB intramuscularly (IM) on day 0. The vaginal device remained for 7 days. On day 6, the cows were injected with 400 IU eCG (IM) and 5 mg Dinoprost (into vulvar submucosa-VS). After removing the vaginal devices (day 7), estrus detection was carried out twice a day during 48 h and the animals were inseminated 12 h after detection (AI). Cows which were not detected in estrus received 100µg of GnRH and were inseminated after 16 to 24 h (TAI). In the eMGPG group, the animals were injected with 400 IU of eCG (IM) on day 0 to increase the follicular growth and GnRH response. After 72 h of eCG, the heifers were injected with 100 ?g of GnRH (IM) and received a vaginal device containing 250 mg of MPA for 7 days. The animals were injected with 5 mg of Dinoprost (SMV) when MPA was removed. The estrus detection, AI and GnRH injection were similar to BioRep group. In the eGPG group, the Ovsynch protocol was used but the heifers were injected with 400 IU of eCG (IM) 72 h before the first injection of GnRH and estrus was detected between injections of Dinoprost and second GnRH injection. The estrus detection rate in BioRep group (52.1%) was higher than that in eMGPG (23.5 %) and GPG (22.3 %; P=0.0001) group. The conception rate did not differ among groups (60.4 %, 65.0 % and 68.4 %). The pregnancy rate was higher in BioRep group (45.6 %) than in eGPG group (28.2 %; P=0.0175), but did not differ from eMGPG group (37.6 %). We conclude that the use of eCG to increase the follicular response to GnRH is not efficient as exogenous gestagen source to synchronize-induce estrus in beef heifers at 12-14 months of age.

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VAGINAL DEVICES (PRID®) MICROBIOLOGICAL EVALUATION AFTER UTILIZATION AND DISINFECTION

Oliveira Filho, B.D.[1]; Gambarini, M.L.[1]; Araújo, I.FL.[2]; Garcia, A.M.[2]; Andrade, M.A.[1]

[1] Animal Production Dept - EV/UFG, Goiânia-GO. bene@vet.ufg.br
[2] Veterinary Medicine undergraduate students - EV/UFG, Goiânia-GO.

Estrus synchronization is an useful tool for cattle management, especially for embryo transfer programs. With new drugs utilization, the technique became more efficient allowing the development of protocols establishment using vaginal devices impregnated with progestagens, which enable ovulation synchronization, eliminating estrus observation, one of the problems in artificial insemination programs. So the elevated costs of the products carried to the reutilization of the devices. The aim of this study was to verify the contamination occurrence and present microorganisms, after the reutilization vaginal devices. One hundred and forty one Nelore cows were submitted to a protocol for fixed time artificial insemination with PRID®, during nine days. After device removal samples were taken with the sterile swabs directly of PRID® surface. Then the devices were immersed in a solution of methyl ammonium chloride 10%, for 20-30 minutes. The samples were maintained in transport medium; in the laboratory they were going chimed in Tioglicolate broth, Selenite Cistein Broth, Blood and Sabouraud agar and incubated to 37° C, for 18-24 hours. The samples in Sabouraud Agar remained in room temperature for 14 days. The Gram negatives and positive bacteria were identified according Baron et al., (1994). For Gram positive cocos identification it was adopted the colony characterization, manitol utilization, oxidation and glucose fermentation. In 92.87% (131) of samples taken before the disinfection there was *bacterial* growth, while after the immersion in methyl ammonium chloride 10% the bacterial growth was verified in 07 (4.96%) samples, showing statiscal significance ($p<0,05$). The more frequent isolated bacteria in pré-disinfection samples were *Escherichia coli, Pseudomonas aeruginosa* and *Corynebacterium pyogenes*, while in the post disinfection samples the most frequent were *E.coli* (n=3) and *P. aeruginosa*, *Staphylococcus spp, S. epidermidis* and Yeast in one sample each. These results allow to conclude that the methyl ammonium chloride solution 10% is efficient to eliminate the contamination originating of the vagina and present in the device, making possible the reutilization of PRID® and reducing the possibility of microorganisms transmission to other cows.

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EFFECT OF DIFFERENT OVULATORY INDUCTION IN SYNCHRONIZATION RATE

Meneghetti, M.[1]; Losi, T.C.[1]; Martins Jr., A.P.[2]; Vilela, E.R.[2]; Vasconcelos, J.L.M.[2]

[1]Aluno do Programa de Pós-Graduação em Zootecnia da FMVZ-UNESP-Botucatu-SP [2]Lageado Consultoria Agropecuária [3]DPEA-FMVZ-UNESP-Botucatu-SP, 18.618-000
vasconcelos@fca.unesp.br

A variety of synchronization protocols have been developed to facilitate and improve AI and ET at fixed time by increasing synchronization rate. The objective of this trial was to evaluate synchronization of ovulation induced by different hormones (ECP, BE and GnRH). Beef cows (n = 946) with 40 to 110 days post-partum and BCS 3.06 ± 0.45 were kept at pasture with mineral *ad libitum*. The animals were submitted to the following protocol: insert of CIDR (pre used for 9 or 18 days) + injection of EB (Estrogin®, 2.0 mg, i.m.) on day 0; CIDR were removed at day 9. Calf removal was performed from day 9 until timed artificial insemination (TAI). On day 9 the animals were divided in four groups: Groups 1 and 2 received ECP (E.C.P.®) at doses of 0.5 or 1 mg, respectively at day 9; Group 3 received 1 mg of EB (Estrogin®) at day 10; Group 4 received 100 µg of GnRH (Fertagyl®) at TAI (48 to 54 hours after CIDR removal). Ciclicity was determined by two ultrasound examinations on days 0 and 7. Animals with luteal tissue in at least one of the exams were considered cycling. Animals with luteal tissue at day 7 received dinoprost trometamina (Lutalyse®, 25 mg, i.m.). The ovulation and CL regression rates were determined by two ultrasound examination at the moment of TAI and 48 hours later. The conception was determined 28 days after TAI by ultrasound. Conception rate was analyzed by logistic regression and the variables included in the model were cyclicity, period of pre used CIDR and hormonal induction of ovulation. There was no treatment effect on synchronization and pregnancy rates and conception rate of cows that were effectively synchronized, respectively: 90.0% (99/110); 58.2% (64/110) and 64.5% (64/99) in G1(0.5mg of ECP), 87.4% (180/206); 46.1% (95/206) and 51.7% (93/180) in G2 (1 mg of ECP); 89.9% (285/317); 52.1% (165/317) and 56.8% (162/285) in G3(EB); 90.1% (282/313); 54.0% (169/313) and 59.2% (167/282) in G4(GnRH). These data suggest that 0.5mg of ECP could be used to induce ovulation due to high synchronization rate and lower cost as compared to GnRH.

EFFICIENCY OF DIFERENT DOSES OF FERTIRELIN ACETATE ON FOLLICULAR DINAMICS AND PROGESTERONE CONCENTRATIONS IN DAIRY COWS SUBMITTED TO THE OVSYNCH PROTOCOL

Gioso, M.M.[1]; Fernandes, C.A.C.[1,2]; Oba, E.[1]; Carvalho, M.A.P.[3]; Oliveira, E.R.[2]

[1] Department of Animal Reproduction and Veterinary Radiology - FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. [2] Biotran LTDA – Alfenas- MG, Brazil. [3] Medicate Veterinarian.
mmgioso@yahoo.com.br

The objective of this work was to compare the efficiency of two different doses from an analog of GnRH (Fertirelin Acetate) about the synchronization of follicular wave emergence, induction of ovulation and progesterone concentration in dairy cows submitted to Ovsynch protocol. Were used seventy seven dairy cows, on 60 to 120 days postpartum and body score of 2.5 to 4.0 (range 1 to 5). The animals were previously submitted to ultrasound evaluation using a linear transducer (6/8MHz, ESAOTE, model Falco-100). Animals presenting a reasonable number of follicles either witch a presence of CL or without CL but witch 10 mm follicles were introduced in the experiment. The females were randomly distributed in four treatments: Group 1 (n=21) - 100 (g (full dose) of Fertirelin (Fertigen®, Schering Plough - Coopers, Brazil) at day 0, PGF2α (500 μ; Ciosin®, Schering Plough - Coopers, Brazil) at day 8 and 100 (g of Fertirelin at day 10. Group 2 (n=21) - 100 (g of Fertirelin at day 0, PGF2α (500 (g) at day 8 and 50 (g of Fertirelin at day 10. Group 3 (n=19) - 50 (g of Fertirelin at day 0, PGF2α at day 8 and 100 (g of Fertirelin at day 10. Group 4 (n=16) - 50 (g of Fertirelin at day 0, PGF2α (500 (g) at day 8 and 50 (g of Fertirelin at day 10. Ultrasound evaluations and blood collections were accomplished on days -1; 0; 2; 8; 10 and 12. Progesterone concentrations were determined by a RIA - radioimmunoassay (DPC Medlab®). Follicular wave induction rates after first Fertirelin administration and induction of ovulation rates after second administration of the same product were compared by Qui-square test. The effects of the treatments in the different days of analysis about the P4 concentrations were submitted by ANOVA. The synchronization rates of the first follicular wave and ovulation rates at the end of the treatments were: 70.0% and 66.2%, respectively, with no difference among the groups (P>0.05). The mean P4 concentrations were similar among treatment groups (p>0.05) on day 0 (1.27 ± 1.63ng/ml), day 2 (1.36±1.73ng/ml), day 8 (1.24±1.31ng/ml), day 10 (0.13±0.20 ng/ml) and day 12 (0.15±0.28ng/ml). The initial increase in the P4 profile demonstrates the effect of Fertirelin on the existent follicles, probably due to ovulation or indirectly luteinization induced by this hormone administration. The decrease in the P4 concentrations after day 8 was attributed to luteolysis induced by PGF2a. The increase on P4 concentrations at the last two days of analysis (days 10 and 12) shows ovulation. Progesterone concentrations, follicular wave synchronization and induction of ovulation demonstrate that Fertirelin was efficient inducing emergency of a new follicular wave, as well as the induction of the ovulation at the end of the protocol, using the dose of either 50μ or 100 μg.

SYNCHRONIZATION OF OVULATION IN ANESTROUS NELORE COWS, TREATED WITH HORMONAL PROTOCOL WITHOUT PROGESTERONE OR PROGESTAGENS

Souza, A.F.[1a]; Pinheiro, V.G.[1a]; Ereno, R.L.[2]; Barros, C.M.[1]

[1]Departamento de Farmacologia, Instituto de Biociências, [2]Departamento de Reprodução Animal, FMVZ-UNESP, Botucatu-SP, Brasil. alfredoferrari@gmail.com

Postpartum anestrous cows are usually treated with progesterone/progestagens protocols in order to induce ovulation and allow pregnancy. In the present work the efficiency of a protocol (modified GPE), in which temporary calf removal (TCR) and/or administration of eCG, replaced the use of progesterone/progestagens, was evaluated in anestrous animals. Anestrous Nelore cows (40 to 60 days postpartum, n=22) were randomly allocated in two groups: GPE/eCG and TCR/GPE/eCG. At random stage of the estrous cycle (D0), animals from Group GPE/eCG were treated with GnRH (50 µg, licereline, i.m. Gestran Plus®) and seven days later (D7) they received PGF2α (150 µg, d-cloprostenol, i.m., Prolise®) and eCG (300 UI, i.m., Novormon®). On D8, estradiol benzoate (EB, 1mg, Estrogin®) was administered, and 30 to 36h afterwards all cows were inseminated at fixed-time (FTAI), without estrus detection. The animals from group TCR/GPE/eCG received the same treatment described above, associated with temporary calf removal (during 48 h) before beginning hormonal treatments. The ovaries were examined by ultrasonography (Aloka SSD 500, 7.5 MHz probe) before (D-12, D-2), during (D0, D7, D8), and after (D9, D10, D11, D17, D60) hormonal treatments. The absence of CL, during the ultrasonography performed before starting the treatments, was the criteria used to classify the cows as in postpartum anoestrus. Administration of GnRH (D0), induced ovulation (determined by the presence of CL on D7) in 6 of 11 cows (54.5%) from Group GPE/eCG and TCR/GPE/eCG. In relation to synchronization of ovulation after EB administration, in Group GPE/eCG, 2 cows (18.1%) ovulated until 12 h after FTAI, and 4 (36.3%) 12 h afterwards (24 h after FTAI). In Group TCR/GPE/eCG, 1 cow (9 %) ovulated approximately 6 h before FTAI, 2 (18.1%) 12 h after FTAI, and 2 (18.1%) 12 h afterwards. Pre-ovulatory follicles had a diameter of 11.0±1.3 and 11.5±2.6 mm, in Groups GPE/eCG and TCR/GPE/eCG, respectively. Pregnancy rates, determined by ultrasonograpy 40 to 46 days after FTAI, were 27.2% (3/11) e 45.4% (5/11), respectively. The results indicate that association of TCR to protocol GPE/eCG did not improve synchronization of ovulation or pregnancy rates ($p>0,005$). However, increase on pregnancy rates was observed in another experiment with a large number of animals, in which cows from Group TCR/GPE/eCG had higher pregnancy rates (51.5%; 34/66) when compared to animals from Group GPE/eCG (28.3%, 21/74; $p<0.05$).

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SYNCHRONIZATION OF BOVINE RECIPIENT'S ESTRUS WITH GnRH AND hCG

Machado, V.C.[1]; Dias, A .J.B.[1]

[1]Laboratory of Improvement Genetic Animal /LMGA/ CCTA/ UENF,
Campos dos Goytacazes-RJ, 28013-600, Brazil. Vtrmachado@bol.com.br

Estrus synchronization is a common practice in the embryos transfer program. Currently the synchronization protocols of estrus for artificial insemination or embryos transfer in fixed time (ETFT) are being based on the use of progesterone dispositive to get such effect, what hardly increases the coast of the protocols. Ahead of the displayed were tried to evaluate the use of a protocol for bovine recipients estrus synchronization based on the use of GnRH and hCG on the size of the formed corpus luteum. Fifteen holstein 3/4 heifers, all cyclical ones with a between 3 and 4 body condition (1 to 5 scale) have been used, and the treatments have been done each 60 days. A commercial protocols (ETFT) have been used as control group (T1). In D0 was placed the progesterone dispositive with 2mg of oestradiol benzoate, in D8 the progesterone dispositive was removed and applied 2 ml analogous of prostaglandine, in D9 was applied the second dose of oestradiol benzoate (1mg), in D17 the ovarium have been evaluated for presence of corpus luteum and quantification of the size through ultrasonography. Animals of the experimental group (T2) have been dealed with the following protocol: D0 was applied analogous of the GnRH (gonadoreline 100mg, PROFERTIL®), in D7 applied analogous of the PGF2α (0.150mg D-Cloprostenol, PROSTAGLANDINA TORTUGA®), D9 applied hCG (1000UI, VETECOR®), in D16 the ovaries have been evaluated for presence of corpus luteum and quantification of the size through ultrasonography. The presence of the C.L. was observed in 83% of the animal of the control group (T1), and in 95.6% of the animals of T2. The average diameter of the C.L. was of 19.7mm for T1 and 17.6 mm for T2. The results demonstrate that the protocol used in T2, with the administration IM of a small concentration hCG (1000 UI) dose were efficient for the formation of corpus luteum in cyclical heifers.

VARIATION ON CONCEPTION RATES IN SUCKLING NELORE COWS SYNCHRONIZED WITH INTRAVAGINAL PROGESTERONE RELEASING DEVICE ASSOCIATED WITH ESTRADIOL BENZOATE OR CYPIONATE

Penteado, L.[1]; Sá Filho, M.F.[2]; Martins, C.M.[2]; Gimenes, L.U.[2]; Ayres, H.[2]; Baruselli, P.S.[2]

[1] ETAPA, Jandaia dos Sul-PR, [2] Departamento de Reprodução Animal, FMVZ/USP, 05508-000, São Paulo-SP, Brasil. luciano@grupocarlosmassa.com.br ou barusell@usp.br

The aim of this study was to evaluate the conception rate of suckling Nelore cows (*Bos indicus*) treated with intravaginal progesterone releasing device (CIDR, Pfizer, Brazil) associated either to two doses (0.5 ou 1.0mg) of estradiol cypionate (EC) at CIDR withdrawal or to 1.0mg of estradiol benzoate (EB) 24h later. A total of 299 postpartum beef cows (60-90 days postpartum) received at beginning of treatment (Day 0) a CIDR associated to 2mg of EB, i.m. (Estrogin, Farmavet, Brasil). On Day 8, CIDR was removed and PGF2α (Lutalyse, Pfizer, Brasil) was administered. The females were homogeneously assigned according to body condition score and postpartum period in three groups: Group 1mgEB (n=99), 1 mg of EB, i.m., 24 hours after intravaginal device withdrawal; Group 0.5mgEC (n=100) or Group 1mgEC (n=100), 0.5 or 1mg of EC (ECP®, Pfizer, Brasil), i.m. at moment of CIDR removal, respectively. All females were submitted to fixed-time artificial insemination (FTAI) 52 to 56 hours after CIDR withdraw, being the ejaculates homogeneously assigned into Groups. Pregnancy diagnosis was performed by ultrasonography 30 days after FTAI. The results were analyzed by Chi-square test. A higher conception rate was observed in Group 1mgEC (55.0%, 55/100)[a] than in Groups 0.5mgEC (41.0%, 41/100)[b] and 1mgEB (38.4%, 38/99)[b]. The administration of 1mg of EC at moment of intravaginal progesterone releasing device withdraw promoted higher conception rates than 0.5mg EC or 1mg EB on Day 9. The ovulation induction using 1 mg of estradiol cypionate in FTAI programs require one less handling of cows and also facilitates its administration due to major drug volume utilized, without compromising the treatment efficiency.

(Acknowledgments: Pfizer)

FOLLICULAR DYNAMICS OF PREPUBERTAL NELORE (*Bos indicus*) HEIFERS TREATED WITH AURICULAR NORGESTOMET IMPLANT AND ESTRADIOL BENZOATE ASSOCIATED OR NOT TO PROGESTERONE INJECTION

Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Madureira, E.H.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo-SP, 05508-000, Brasil.
barusell@usp.br

The follicular dynamics in prepubertal Nelore (*Bos indicus*) heifers treated estradiol benzoate (EB) associated or not to progesterone injection (P4) at beginning of the treatment with auricular Norgestomet implant (Day 0) was evaluated. Eighteen prepubertal Nelore heifers (n=18, absence of corpus luteum detected by ultrasonographic examinations on Day –28, Day -14 and at the beginning of the treatment) were randomly assigned in two groups (EB ou EB+P_4). On Day 0, the females received an auricular Norgestomet implant (Crestar®, Intervet, Brazil). The EB Group received 2mg of EB i.m. (Estrogin®, Farmavet, Brazil), while the EB+P_4 Group received 2mg of EB plus 50mg of P4 i.m. (Index Farmacêutica, Brazil). On Day 8, the implants were withdraw plus $PGF_{2\alpha}$ i.m. (Preloban®, Intervet, Brazil) and 1mg of EB were administered 24 hours later. The diameter of dominant follicle on Day 0 (DF0), the moment of the new follicular wave emergence (FE), diameter of dominant follicle at Day 8 (DF8), the maximum diameter of dominant follicle (DFMAX), the ovulatory rates (OR) and the moment of ovulation after implant removal (OVM) were evaluated by ultrasonographic examinations. The data were analyzed by Anova and Chi-square tests in SAS. The groups presented ovulations after EB administration at beginning of treatment [EB (22.2%; 2/9) and EB+P_4 (44.4%; 4/9)]. The results of the heifers treated with EB vs. EB+P_4 were: DF0 (1.12±0.05 vs. 1.05±0.04 cm; $p>0.05$), FE (3.33±0.33 vs. 2.67±0.19 days; $p>0.05$), DF8 (0.86±0.07 vs. 1.01±0.07 cm; $p>0.05$); DFMAX (1.05 ± 0.05^a vs 1.26 ± 0.06^b cm; $p<0.05$); OR [(100.0% (8/8) vs. 77.7% (7/9); $p>0.05$]; OVM (73.7±1.7 vs 72.0±0.0h; $p>0,05$). The results suggests that is possible obtain satisfactory rates of the synchronized ovulation in Nelore heifers treated with EB or EB+P4 at beginning of the treatment with auricular Norgestomet implant. Further studs should be done to evaluate the conception rates in IATF in Nelore prepurbetal heifers utilizing this protocols.

Acknowledgments: Intervet

EFFECT OF PGF2α ADMINISTRATION IN THE BEGINNING OF AURICULAR NORGESTOMET IMPLANT TREATMENT ON FOLLICULAR DYNAMICS OF NELORE HEIFERS (*Bos indicus*)

Torres-Júnior, J.R.S.[1]; Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Madureira, E.H.[1]; Baruselli, P.S.[1]

[1] Departamento de Reprodução Animal, FMVZ-USP, São Paulo-SP, 05508-000, Brasil.
barusell@usp.br

The present study aimed to evaluate the effect of PGF2α administration in the beginning of auricular Norgestomet implant treatment on follicular dynamics of Nelore heifers (*Bos indicus*). Twenty two cycling heifers (20 to 24 months and > 325kg) were presynchronized with two PGF2α doses i.m. (Preloban®, Akzo Nobel, Intervet, Brazil), with 14 days interval (D-28 and D-14). On Day 0, all heifers received a 3 mg auricular Norgestomet implant (Crestar®, Akzo Nobel, Intervet) plus 2 mg of estradiol benzoate i.m. (EB; Estrogin®, Farmavet, Brazil). At this time, PGF2α Group (n=11) received a PGF2α dose, while Control Group (n=11) did not received additional treatment. On D8 the implant was removed and PGF2α was administered and, 24 hours later (Day 9), 1 mg of EB was injected in all heifers. Follicular diameters were monitored on Day 0 (∅D0), Day 8 (∅D8), maximum follicular diameter (∅MAX) and ovulatory follicular diameter (∅OV), moment of ovulation (MOV) and ovulation rate (ROV) by transrectal ultrasonographic scanning. Data were analyzed by ANOVA test, on SAS® program. There was no effect of treatment with PGF2α on the appraised parameters (P>0,05). The results of PGF2α and Control Groups were respectively: **∅D0**: 0.97±0.07 vs. 1.05±0.05; **∅D8**: 0.98±0.05 vs. 1.02±0.05; **∅OV**: 1.17±0.05 vs. 1.26±0.03; **∅MAX**: 1.21±0.06 vs. 1.30±0.03; **MOV**: 69.60±2.40 vs. 72.00±0.00 hours; **ROV**: 91.0% (10/11) vs. 82.0% (9/11). It can be concluded that it is possible to get satisfactory ovulation rates in Nelore heifers using auricular Norgestomet implant plus estradiol benzoate, without luteolysis induction in the beginning of treatment.

Acknowledgments: Intervet

TREATMENT WITH hCG PLUS LH, USED TO INDUCE OVULATION IN SUPERSTIMULATED COWS, DOES NOT ALTER EMBRYO PRODUCTION NOR PREGNANCY RATES

Nogueira, M.F.G.[1*]; Buratini Jr., J.[2]; Barros, C.M.[1]

Departments of [1]Pharmacology and [2]Phisiology, Instituto de Biociências, UNESP, Botucatu, São Paulo, 18618-000, Brazil.

The events following the LH surge are related to the presence of LH receptor (LHR) and its affinity to LH. Studies on LHR gene expression in theca and granulosa cells from bovine follicles have demonstrated the presence of, at least, 4 isoforms of LHr mRNA by alternative splicing. Two of those detected isoforms could be translated to functional proteins with different affinities to their ligands. One of them (full-length) has affinity to both LH and hCG molecules, whereas the other isoform (with deletion of exon 10) has affinity to hCG only. Based on this information, the present study tested the hypothesis that, in cows superstimulated with FSH, administration of both LH and hCG, as an attempt to stimulate any variety of LH receptor present in the follicles, would result in improvement of oocyte quality and/or increase in ovulation rate. Nelore cows (*Bos taurus indicus*) were superstimulated according to the protocol coined P-36 (Barros CM *et al*. 2003 Theriogenology **59**, 524 abst), and embryos were flushed 7 to 8 days after inducing ovulation. Ovulation was induced with LH (pLH, 12.5 mg, i.m., Lutropin, Vetrepharm, Ontario, Canada, Group 1) or both LH (12.5 mg) and hCG (1500 IU, i.m., Chorulon 5000, Intervet, The Netherlands, Group 2). Superstimulation protocol and embryo transfer were performed simultaneously on both groups. Mean of total structures, viable embryos and viability rate were, respectively: 14.6; 10.2 and 69.5% (Group 1, n=65 flushings) and 12.5; 9.8 and 78.2% (Group 2, n=26). There was no significant difference between groups (P>0.05). The pregnancy rates of transferred embryos (excellent, good, fair and poor qualities) were 42.0% (266/634; Group 1) and 41.5% (88/201; Group 2, P>0.05). When pregnancy rates were analyzed separately for each embryo quality, embryos of excellent, good, fair and poor qualities yielded, respectively, 50.0; 42.8; 35.0 and 18.2% (Group 1) and 43.7; 45.7; 35.0 and 26.7% (Group 2). It is concluded that simultaneous administration of LH and hCG to induce ovulation in superstimulated animals did not alter neither the production of viable embryos nor the pregnancy rates after embryo transfer. *Fellowship from FAPESP.

EFFECT OF LENGTH OF INTRAVAGINAL PROGESTERONE DEVICE EXPOSURE ON PREGNANCY RATES IN BEEF HEIFERS

Gattass, C.B.A.[1]; Sá Filho, O.G.[2]; Vasconcelos, J.L.M.[2]

[1] Agropecuária JB, Miranda-MS. [2]Departamento de Produção e Exploração Animal – FMVZ/ UNESP, Botucatu-SP, 18618-000, Brasil. vasconcelos@fca.unesp.br

A limiting factor of TAI in large herds of cows is the fact that animals are kept long time at management facilities, submitted to stress and inseminated out of ideal moment. Physical limitations of the technicians after manipulate many animal's cervix is also an important factor. Otherwise, manage a higher number of animals in a lower number of days is a way of reduce costs per treated animal, because labor is maximized. The objective of this study was to verify if anticipating one day of intravaginal progesterone device (CIDR®) withdrawal changes the efficiency of the synchronization of ovulation protocol proposed by Sá Filho et al. (2003; Rev. Bras. Reprod. Anim., 27:430), allowing use strategies of management in which treatments are begun in large number of animals and half of them are inseminated in a day and the other half by the day after. 333 crossbred heifers (Nellore x Angus), body condition score (BCS) between 5 and 7 (1-9 scale) and age between 18 and 24 months, received CIDR® + Estradiol Benzoate (EB; Estrogin® 2mg i.m.) at Day 0 and a PGF2α injection (Lutalyse® 12.5mg i.m.) at Day 7. After this moment, the heifers were randomly divided in two groups with CIDR® been removed at Day 8 (Group 1, n=152) or at Day 9 (Group 2, n=181). In both groups, an EB injection (0.8 mg) was given 24 hours after devices withdrawal (Day 9 in Group 1 and Day 10 in Group 2) and heifers were inseminated 30-36 hours after EB injection (Day 10 in Group 1 e Day 11 in Group 2). 17-25 days after TAI heat was watched and animals returning on estrous were inseminated after 12 hours. Pregnancy checks were done by rectal palpation 40 and 65 days after TAI. There was no difference between treatments regarding to pregnancy rate at TAI (38.46% vs. 41.18% for Groups 1 and 2, respectively; P=0.69) and pregnancy at + return (66.35% vs. 57.84% for Groups 1 and 2, respectively; P=0.21). Either, no effect of BCS on pregnancy rate at TAI was detected (38.99; 40.13; and 39.69% for scores 5, 6 e 7, respectively; P=0.18). These data suggest that both treatments (CIDR for 8 and 9 days) provide high synchronization rates, showing that is possible to work with large amount of animals, in a manner that treatment is begun for the whole herd at the same day and AIs are done in two different days with similar results.

CYCLICITY AND eCG TREATMENT EFFECTS ON FOLLICULAR DYNAMICS AND CONCEPTION RATE IN NELORE HEIFERS TREATED WITH NORGESTOMET AURICULAR IMPLANT AND ESTRADIOL BENZOATE

Sá Filho, M.F.[1]; Penteado, L.[2]; Reis, E.L.[1]; Gimenes, L.U.[1]; Baruselli, P.S.[1]

[1] Departamento de Reprodução Animal, FMVZ/USP, São Paulo-SP, Brasil, [2] ETAPA, Jandaia do Sul-PR. barusell@usp.br

A total of 177 Nelore heifers were selected according to *corpus luteum* (CL) presence (Cycling) or absence (Ncycling) at the beggining of treatment (Day 0). The heifers were homogeneously assigned in four groups according to cyclicity and to eCG treatment at the Norgestomet implant withdrawal (2x2 factorial). On Day 0, all females received a Norgestomet auricular implant (Crestar®, Intervet, Brasil) plus 2 mg of estradiol benzoate i.m. (EB, Estrogin®, Farmavet, Brasil). On Day 8, implants were removed and PGF2α, i.m. (Preloban®, Intervet, Brasil) was administered. The eCG Groups received 400 IU of eCG, i.m. (Folligon®, Intervet, Brasil) on Day 8. One mg of EB was injected on Day 9 in all heifers, which were fixed-time inseminated (FTAI) 30-34 hours after EB administration. Ultrasonographic scanning was performed to evaluate dominant follicle diameter on day 8 (DF8), ovulation rate (OVR; CL presence five days after FTAI), CL diameter five days after FTAI (CLD), conception rate 30 days after FTAI (CR) and conception rate in ovulating heifers (CROVU). Data were analyzed by Anova and Chi-square test. No interactions were observed among treatments. The main effects of Without eCG vs. With eCG and Ncycling vs. Cycling treatments were, respectively: [DF8 (0.82±0.02 vs. 0.86±0.02) and (0.89 ± 0.02^{a} vs. 0.82 ± 0.02^{b}), OVR (67.8%; $59/87^{a}$ vs. 86.7%; $78/90^{b}$) and (81.1%; 43/53 vs. 75.8%; 94/124), CLD (1.38 ± 0.03^{a} vs. 1.55 ± 0.03^{b}) e (1.42 ± 0.04^{a} vs. 1.51 ± 0.02^{b}), CR (36.8%; $32/87^{a}$ vs. 50.0%; $45/90^{b}$) and (30.2%; $16/53^{a}$ vs. 49.2%; $61/124^{b}$) and CROVU (54.2%; 32/59 vs. 57.7%; 45/78) and (37.2%; $16/43^{a}$ vs. 64.9%; $61/94^{b}$); $a \neq b$, $p<0.05$]. Data are suggestive that is possible to get satisfactory results in cycling animals and that eCG treatment increases the conception rates in Nelore heifers treated with Norgestomet auricular implant and estradiol benzoate.

Acknowledgements: Intervet

FOLLICULAR DYNAMICS IN *Bos indicus* HEIFERS TREATED WITH NORGESTOMET AURICULAR IMPLANT OR PROGESTERONE INTRAVAGINAL RELEASING DEVICE

Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Ayres, H.[1]; Carvalho, J.B.P.[2]; Carvalho, N.A.T.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo-SP; [2]Instituto de Zootecnia, Pindamonhangaba-SP. barusell@usp.br

In the present study 48 cycling heifers were pre-synchronized with two PGF2α doses (Day-24 and Day-12) and assigned in four groups according to progesterone (P4; CIDR) or progestin (Crestar) source and with or without PGF2α administration at the beggining of treatment (Day 0; 2 x 2 factorial). On Day 0, 2 mg of estradiol benzoate i.m. (EB; Estrogin®, Farmavet, Brazil) was administered in all animals. The CIDR Group received an P4 intravaginal releasing device (CIDR®, Pfizer, Brazil), while CRESTAR Group received an Norgestomet auricular implant (Crestar®, Intervet, Brazil). The CIDR+PGF and CRESTAR+PGF Groups received an additional PGF2α dose i.m. (Preloban®, Intervet, Brazil) on Day 0. On Day 8, implants or devices were withdraw plus PGF2α administration, and on Day 9, 1mg of EB was injected. Follicular dynamics was evaluated by ultrasonography each 24 hours from Day 0 to Day 8 and every 12 hours from Day 9 to Day 12. Data were analyzed by Anova and Chi-square tests and the specifically the variable widespread of ovulation by Bartlett test. No interactions were observed being the main effects of treatments CIDR vs CRESTAR and WITH vs. WITHOUT PGF, respectively: DF Ø on Day 0 (1.01±0.03 vs. 1.00±0.04; 0.99±0.03 vs. 1.02±0.04 cm), moment of follicular emergence (3.1±0.15 vs. 2.9±0.13; 3.1±0.12 vs. 2.91±0.15 dias), DF Ø on Day 8 (0.83 ± 0.03^{a} vs. 0.99 ± 0.03^{b}; 0.88±0.04 vs. 0.94±0.03 cm), maximum DF Ø (0.97 ± 0.04^{a} vs 1.17 ± 0.04^{b}; 1.03±0.05 vs. 1.13±0.04 cm), follicular growth rate (0.10 ± 0.01^{a} vs. 0.13 ± 0.01^{b}; 0.11±0.01 vs. 0.12±0.01 cm/day), ovulatory rate [57.1% $(12/21)^{a}$ vs. 83.3% $(20/24)^{b}$; 63.6% (14/22) vs. 78.3% (18/23)] and moment of ovulation after implant/ device removal (79.6 ± 2.92^{ax} vs. 73.2 ± 0.83^{by}; 77.1 ± 2.42^{x} vs. 74.1 ± 1.14^{y} hours; a≠b, P<0.05; x≠y, P<0.05 in Bartlett test). It was verified effect of PGF2α use in the beginning of treatment only on ovulation synchronization. However, Norgestomet auricular implant treatment improved the follicular growth rate, dominant follicle diameter, ovulatory rate and ovulation synchronization when compared to P4 intravaginal device in *Bos indicus* heifers.

Acknowledgements: Intervet

EFFECT OF DIFFERENT LEVELS OF CONCENTRATE SUPPLEMENTATION DURING POS-PARTUM AND THE EFFECTS IN OVARIAN DYNAMICS OF DAIRY COWS

Rigo, A.G.[1]; Salmazo, R.[2]; Schroeder, R.V.[1]; Mizubuti, I.Y.[2];
Costa, M.C.[1]; Seneda, M.M.[1]

[1] Department of Veterinarian Clinic and Reproduction - State's University of Londrina, Londrina-PR, 86051-990, Brazil. [2] Department of Zootecnia- State's University of Londrina, Londrina-PR, 86051-990, Brazil. agrigo@pop.com.br

Among the critical aspects of milk production and reproductive performance, the nutritional factor has an expressive impact due to the negative energetic balance . The aim of this work is to study different levels of diet supplementation during the post-partum period and their effect on the reproduction of dairy cows. The treatments (T), had been distributed according to the level of supplied concentrate: T1 (n=9) 1 kg of concentrate for every 2,5 kg of milk produced; T2 (n=9) 1 kg of concentrate for every 2.0 kg of milk produced and T3 (n=9) 1 kg of concentrate for every 1.5 kg of milk produced, starting after birth. A commercial concentrate was used which presented 75% of total possible digestive nutrients (NDT) and 18% of rude protein (PB). The animals were submitted to the Trans-rectal ultrasound evaluation weekly, for evaluation of corpus luteum, besides the number and diameter of the visualized follicle. Daily the animals were inspected for heat detection. After 44 weeks and 242 ultrasound evaluation, it was observed a smaller diameter of the greater follicle in T2 (13.77mm) compare to T1 (15 mm) and T3 (16.62 mm). Nevertheless, to first heat detection, group T2 present smaller average in the break birth-first heat (36 days) compare to T1(41 days) and T3 (50 days). These preliminary results suggest a bigger efficiency to T2 about heat detection, in spite of smaller diameter from biggest follicle in the animals in this group. It is emphasized that more precise conclusions will be possible once the work is concluded.

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SYNCHRONIZATION OF OVULATION FOR FIXED TIMED ARTIFICIAL INSEMINATION DURING THE REPRODUCTIVE SEASON IN CROSSBRED EWES

Castilho, C.; Mori, M.M.; Tomé, R.S.

Faculdade de Medicina Veterinária, UNOESTE, Campus II, P. Prudente – SP, 19067-175.
calie@unoeste.br.

The objective of the present experiment was to evaluate the pregnancy rate in ewes after synchronization of ovulation with progestagen, PGF2α, and eCG (equine chorionic gonadotrophin) and fixed-time artificial insemination (IATF) with fresh diluted semen deposited at the external cervical os. Fifty-three crossbred ewes were used during the reproductive season (June). The ewes were kept at pasture (*Brachiaria*) and moved into a barn during the night for mineral salt supplementation. Ovulation synchronization treatments were initiated at a random stage of the estrous cycle. Intravaginal sponges containing 50 mg medroxiprogesterone acetate were inserted (day 0) concomitant with treatment with 3,75 mg PGF2α (D-Cloprostenol, Preloban®, Intervet, Holanda). On day 6.5 the ewes were treated with 300 UI eCG (IM; Folligon®) and intravaginal sponges were removed on day 8. Artificial inseminations were performed approximately 48 and 72 h after sponges removal. For insemination, semen was collected at the property, evaluated for viability, mixed with 30 mL of glycine-egg yolk diluent, and 0.5 mL of diluted semen (at least 250 million spermatozoa per each dose) was deposited at the external cervical of each ewe. Pregnancy diagnosis was performed approximately 60 days after IATF using a Pie Medical® ultrasound scanner equipped with a 5,0 mHz linear transrectal probe. A total of 18 ewes became pregnant after IAFT (34%) and another 7 ewes became pregnant after natural breeding in the first heat after IAFT, resulting in a total of 25 ewes pregnant (49%) in a period of approximately 30 days. We conclude that the protocol was effective in synchronizing ovulation and optimizing the use of the ram, however, additional experiments are required to improve the results obtained with IATF in ewes.

Acknowledgments: Intervet Brazil.

INDUCTION OF OVULATION WITH GnRH OR ESTRADIOL BENZOATE AND TWO-DAY OR FIXED-TIME ARTIFICIAL INSEMINATION IN ESTRUS INDUCTION PROTOCOLS FOR POSTPARTUM BEEF COWS

Siqueira, L.C.; Oliveira, J.F.C.; Loguercio, R.S.; Kurtz, H.; Gonçalves, P.B.D.

Universidade Federal de Santa Maria, Departamento de Clínica de Grandes Animais Laboratório de Biotecnologia e Reprodução Animal - BioRep, Santa Maria, RS, Brasil. bayard@biorep.ufsm.br

This experiment was design to compare two estrus induction protocols for cows in postpartum period, using GnRH and two-day artificial insemination (AI) or estradiol benzoate (EB) and fixed-time artificial insemination (TAI). A total of 250 suckled beef cows (50-70 days PP), *Bos taurus*, in a body condition score of 3 (Extremely thin cows were assigned a score of 1 and extremely fat cows, a score of 5) was used. These animals received a vaginal device containing 250 mg of medroxyprogesterone acetate (MPA) and an injection of 5 mg of EB intramuscularly (IM) on day 0. The vaginal device was removed on day 7. On day 6, the cows were injected with 400 IU eCG (IM) and 5 mg Dinoprost (into vulvar submucosa) and calves were removed for 96 hours. After removing the vaginal devices (day 7), the cows were divided in two groups. In BioRep group (n=150), estrus detection was carried out twice a day during 48 h and the animals were inseminated 12 h after detection. Cows which were not detected in estrus received 100μg of GnRH and inseminated after 16 to 18 h (TAI). In EB group, cows were injected im with 1 mg of EB on day 8 (24 h after removing MPA) and were inseminated (TAI) on day 9 (54 h after removing MPA). The diagnosis of pregnancy was performed 40 days after AI with ultrasound. The data were analyzed by Chi-square test. The pregnancy rate was higher ($p<0.01$) in BioRep group (54.7%) than EB group (33.3%). In situations that high pregnancy rate is required, the estrus induction protocols for postpartum beef cows should use two-day AI and GnRH to induce ovulation instead of EB with TAI.

This work was supported by CNPq and FAPERGS.

SYNCHRONIZATION OF OVULATION IN PRE-PUBERTAL NELORE AND BONSMARA HEIFERS, TREATED WITH PROGESTERONE RELEASE INTRAVAGINAL DEVICE (PROTOCOL PEPE)

Ereno, R.L.[1]; Pegorer, M.F.[2]; Nogueira, M.F.G.[1]; Barros, C.M.[2]

[1]Depto. de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, [2]Depto. de Farmacologia – IBB/UNESP, 18618-0000 Botucatu-SP, Brasil. erenoronaldo@ig.com.br

Hormonal treatments with progesterone/progestagens have been used to induce pregnancy in postpartum anestrous cows. In the present work the efficiency of a hormonal protocol with progesterone, to induce synchronization of ovulation, was evaluated in indicus (Nelore, n=14) and taurus (Bonsmara, n=14) heifers, both breeds adapted to the high temperatures found in tropical regions. Pre-pubertal heifers (18 months old), weighting 321.8 ±. 5.8 kg, and body condition score above 3.5 (in a scale from 1 to 5), were treated with protocol PEPE (Progesterone-Estrogen-Prostaglandin-Estrogen). At random stage of estrous cycle all animals received an intravaginal device with progesterone (1.0 g, DIB®, Syntex, Argentina) and 2.0 mg of estradiol benzoate (EB, i.m., Estrogin®, Farmavet, Brazil). Eight days later (D8) heifers were treated with PGF2α (150 mcg d-cloprostenol, i.m., Prolise®, ARSA, Argentina), and DIB was removed. Twenty four hours after DIB removal, animals received EB (0.75 mg, i.m), and 30 to 36 h later all heifers were FTAI, without estrus detection. The ovaries were observed by ultrasonograpy (Aloka SSD 500, 7.5 MHZ probe) at days 0, 8, 9, at the time of FTAI (D10) and 12 h afterwards, in order to evaluate follicular diameter and ovulation rate. The absence of corpus luteum during ovarian ultrasonography confirmed that heifers were pre-pubertal. The size of dominant follicles from Nelore and Bonsmara were, respectively: 7.35±1.48 and 9.07±3.26 mm (D8), 9.10±1.57 and 11.14±3.10 mm (D9) and 9.65±1.43 and 11.54±2.79 mm (D10). Ovulatory follicles had a diameter of 8.5±1.22 mm (Nelore) and 11.5±3.01 mm (Bonsmara), just before FTAI (D10). One Nelore heifer (7.1%) ovulated before FTAI and 4/14 (28.6%) ovulated until 12 h after FTAI (synchronized ovulation). At the time of FTAI, 2 Bonsmara heifers had already ovulated, and 7/14 (50%) ovulated until 12 h after FTAI (synchronized ovulation). The pregnancy rates after FTAI were 7.1% (1/14) for Nelore, and 35.7% (5/14) for Bonsmara heifers. The results indicate that protocol PEPE was more effective (synchronized ovulation) in pre-pubertal Bonsmara than in Nelore heifers.

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EFFICIENCY OF D-CLOPROSTENOL FOR ESTRUS SYNCHRONIZATION AT DIFFERENT DAYS OF ESTRUS CYCLE.

Cembranelli, M.A.R.[1]; Fernandes, C.A.C.[2,3]; Gioso, M.M.[2]; Figueiredo, A.C.S.[2,3]; Oliveira, E.R.[3]; Viana, J.H.M.[4]

[1]Mestrando Ciência Animal – Unifenas; [2]Prof. Faculd. Med. Vet. Unifenas – [3]Biotran LTDA. [4]Embrapa-Gado de Leite. Rua Tatuin, 93, Resid. Teixeira. 37130-000 – Alfenas MG – cacf@biotran.com.br

The bovine estrus synchronization by luteolytic agents as F2α prostaglandin (PGF2α) or its analogous ones, have been widely used in reproductive programs and embryos transfer (Baird, 1992). The objective of this study was to verify the efficiency of sodic cloprostenol (IM) at different phases of the estrous cycle (diestrus) in crossbred cows and heifers. 293 applications of D-Cloprostenol had been carried for estrus synchronization in crossbred heifers or cows used as recipients in a embryos transfer program. The animals had been previously observed in natural estrus and divided in 4 groups according the estrous cycle day. Group A: day 6 to the 8; group B: day 9 to the 11; group C: 14 day 12 to and group D: day 15 to the 17 of the estrous cycle. All the groups had received 150μg (2ml) of D-Cloprostenol IM. After treatment the animals had been kept in the presence of teasers and had been submitted the visual observations three times a day. The standing was indicative of estrus. The efficiency of the synchronization in each group was compared by the χ^2. The general efficiency of the synchronization was of 77.15%. Similar to results described for other studies with the same protocol (Fernandes et al, 1997). The efficiency for groups A, B, C and D was of 66,67[a]; 78.43[b]; 75.58[b] and 78.43%[a] ($P<0.05$), respectively. Animals at initial periods of the estrous cycle showed minor sensitivity to the synthetic analogous of the PGF. The day of the estrous cycle interfere with D-cloprostenol sensitivity.

BAIRD, D.T. Animal Reprod. Science, 28,3, 95-102, 1992.
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STUDY OF SOME ASPECTS THAT CAN INTERFERE ON THE ZEBU FEMALES PREGNANCY IN SYNCHRONIZED OVULATION PROGRAM FOR FIXED TIME INSEMINATION

Bittencourt, R.F.[1]; Ribeiro Filho, A. de L.[1]; Chalhoub, M.[1]; Alves, S.G.G.[1]; Biscarde, C.E.A.[1]; Almeida, A.K.[1]; Portela, A.P.M.[1]; Freitas, D.S.[1]; Silva, A.A.B.[1]; Santana, R.C.M.[1];

[1]Escola de Medicina Veterinária – UFBA, Salvador-BA, 40170-110, Brasil. rfbvet@yahoo.com.br

A group of two hundred and fifty zebu females were divided in four experimental groups. Before the beginning of the experiment, all the animals were evaluated as for the body condition score (BCS). The aim of this study was to verify some factors that can affect the pregnancy rate of zebu females submitted to protocols of synchronized ovulation for fixed time insemination (FTI). In the group 1 (G1 – n=102), the animals received 3mg de Norgestomet (NOR) through auricular implant, on random stage of oestrous cycle on the Day 0. Right after it, an IM injection of 3mg of oestradiol valerate (OV) plus NOR (5mg) was administered. In the Group 2 (G2 – n=20) and Group 3 (G3 – n=78) animals, the same protocol was used, although, right after the auricular implant application, in the G2, it was administered 4,5mg of NOR and 7.5mg of OV. In the G3, the auricular implant was used again (for the second time) and the concentrations administered IM were similar to G2's. In the Group 4 (G4 - n=50) the animals received 3mg of Norgestomet (NOR) in auricular implant, in random stage of oestrous cycle on the Day 0 (D0). Right after it, an IM injection of 2mg of oestradiol benzoate (OB) plus 50mg of progesterone (P4) was administered. A new dose of OB (1mg) was injected to G4 animal on D10. Nine days after (D9), the implants were removed and all the animals were inseminated at a fixed time (FTI), approximately 56h after the implant removal (D11). It was observed the intervals between the beginning of the hormonal treatment (introduction of auricular implant) and fixed time insemination (FTI), introduction of auricular implant and its removal, implant removal (IR) and oestrous observation (OEB), IR and FIT and, finally, OEB and IATF. Statistical analyses were performed using the SAS software version 6.1. The diverse intervals observed didn't have any influence on the pregnancy rate ($P>0.05$), when studied with the population as a whole. When the study was done based on the protocol, in the G1, it was observed the positive correlation between the interval of time until the implant administration and FIT, and the pregnancy rate ($r=0.24$; $P<0.02$), as well as between the pregnancy rate and the interval from IR to FIT ($r=0.23$; $P\leq0.02$). On that group, it was observed, among all the other ones, the smallest interval between IR and FIT (52,17h). Thus, it is possible to consider that the pregnancy rates could be implemented delaying the FITs moment. It was also observed an important correlation between BCS and pregnancy rate of the females in study ($r=0.52$; $P<0.0001$), where the higher the BCS, the greater was the pregnancy rate obtained. The correlations between the CSR and the pregnancy rate were for G1, G2, G3 and G4, respectively, of $r=0.59$ ($P<0.0001$); $r=0.74$ ($P<0.0001$); $r=0.48$ ($P<0.0001$) and $r=041$ ($P<0.01$). Thus, it is shown the importance of the BCS on the pregnancy rate of zebu females submitted to different synchronization protocols for FIT.

ESTRUS BEHAVIOR AND OVULATION RATE IN NELORE FEMALES SYNCHRONIZED WITH PROGESTIN, ESTRADIOL AND EQUINE CORIONIC GONADOTROPHIN

Bergamaschi, M.A.C.M.[1]; Vicente, W.R.R.[1]; Barbosa, R.T.[2]; Machado, R.[2]; Ibiapina, B.T.[3]; Baruselli, P.S.[3]; Alencar, M.M.[2]; Binelli, M.[3]

[1]Universidade Estadual Paulista –UNESP– Campus de Jaboticabal, Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Jaboticabal, SP. [2]Embrapa Pecuária Sudeste, São Carlos, SP. [3]Universidade São Paulo – USP - Departamento de Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia. brunaibiapina@uol.com.br

Detection of estrus in *Bos indicus* is challenging due to the peculiarities of this sub-species and could limit the wide application of artificial insemination (AI). Objective of this study was to evaluate the effect of a protocol to synchronize ovulations using a progestin associated with estradiol valerate in the presence or absence of equine chorionic gonadotrophin (eCG) on preovulatory follicle diameter, ovulation rate and estrus behavior to the synchronization protocol and to the next spontaneous estrus. Sixty lactating Nelore females, approximately 158 days postpartum, received an ear implant containing norgestomet (CRESTAR®, Intervet) and an estradiol valerate injection. Nine days later, implants were removed and animals were randomly assigned to receive 0 or 400 UI of eCG. Follicular dynamics were monitored by ultrasonography from 48 hours after implant removal to the subsequent natural ovulation. Heifers were observed for estrus daily with the aid of teaser bulls. There was no effect of eCG treatment on ovulation rate (70.00 % for both groups), ovulation time (80.00 ± 2.53 h after implant removal for both groups) or synchronized preovulatory follicles diameter (13.38 ± 0.39 e 12.72 ± 0.39 mm for the groups receiving 0 or 400 UI of eCG, respectively; P>0,1). Interovulatory intervals of control (21.38 ± 0.57 dias) and eCG-treated cows (21.75 ± 0.57 dias) were similar. The 60% of cows that did not ovulate in the subsequent estrous cycle exhibited estrus behavior. Ovulation without estrous signs also occurred, both in synchronized estrous cycle (42.86% in both groups) and in the spontaneous, subsequent cycle (44.44% in the Control group and 22.22% in the eCG-treated group). Thus, fixed-time artificial insemination procedures sustained the possibility of breeding without the necessity of observation of estrus behavior and can ensure as much as 70.00% of cows submitted to these protocols. In addition, eCG increased the expression of estrous behavior on the estrous cycle subsequent to synchronized ovulation, which may facilitate breeding of animals that did not conceive in response to the synchronization protocol.

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REUTILIZATION OF NORGESTOMET IMPLANTS IN NELORE COWS.

Almeida, A.B.[1]; Madureira, E.H.[1]; Binelli, M.[1]; Rossa, L.A.[1]; Pimentel, J.R.V.[1]; Bertan, C.M.[1]; Marques, V.M.[1]

[1]Departamento de Reprodução Animal - alexbar@usp.br - Av. Duque de Caxias Norte, 225 - FMVZ - VRA - Pirassununga, SP, Brasil - 13635-900.

Pregnancy rates (PR) of Nelore cows (*Bos taurus indicus*) implanted with new or used norgestomet implants associated to estradiol valerate (EV) or to estradiol benzoate (EB) were compared. Two hundred and forty one lactating cows were divided to receive a 3 mg norgestomet ear implant (n=122) or a previously used norgestomet implant (n=119) for 10 days. Each group was then further divided to receive either a 3 mg injection (IM) of norgestomet associated to 5 mg EV or an injection of 50 mg progesterone associated to 2 mg EB. All animals received an injection of a PGF2α analog (IM) and 24 h after 1.0 mg EB (IM). Time artificial insemination was performed (TAI) 54-56 h after implant removal. The results were analysed by Statistical Analysis System (SAS). The variables PR-TAI and PR-final were submited by PROC GLM according kind of implant (new or used), kind of ester (valerate or benzoate), Breeding season, Parturition (primiparous or multiparous), Body condition and Ovarian condition. There was no implant by estradiol ester interaction ($p<0.05$) in PR at TAI (PR-TAI) or at the end of the breeding season (PR-final). The PR-TAI were similar for animals receiving new or reutilized implants (48.3 vs 48.7%) and for animals receiving both EV and EB (49.5 vs 47.5%). Similar results were found in PR-final for new or used implants (85,2 vs 86,5%) and according to se of EV or EB (86.5 vs 85.2%). It was concluded that PR of lactating Nelore cows were adequate and similar when animals were treated with either new or reutilized norgestomet implants associated with either EV or EB.

EFFECT OF THE REUSAGE OF INTRAVAGINAL DEVICE CRONIPRES® SUPLEMENTED WITH RINGS OF PROGESTERONE IN NELORE COWS INSEMINATED AT FIXED TIME.

Duarte, R.A.[1]; Kozicki, L.E.[1]; Bó, G.A.[2]; Gonçalves, R.[3]; Rosa, S.D.[4]

[1]UFPR, Curitiba/PR, Brasil., [2]Inst. de Reprod. Animal Córdoba, Córdoba, Argentina., [3] Biogenesis Brasil, Curitiba/PR, Brasil., [4] Agropecuária Cruzeiro, Francisco Beltrão/PR, Brasil.
radaduarte@ig.com.br.

This expirement had as objective to evaluate the reusage of intravaginal progesterone device (PD4) Cronipres® (Biogenesis) suplemented with rings that contain 100mg of progesterone (P4), on the pregnancy rates in lactating cows that underwent fixed time artificial insemination (FTAI). One hundred and fifty eight lactating Nelore cows were used (55-70 days after birth), kept in a pasture in Francisco Beltrão-PR. The animals were separated in five groups (factorial 2x2) according to body conditions. On the 0 day, all of them received 2mg of estradiol benzoate EB IM (Estrogin®, Farmavet) and a PD4 (Cronipres®, Biogenesis) reused according to the group they belonged. On the 8th day, PD4 was removed and it was administered 0.15 mg of d-cloprostenol (Croniben®, Biogenesis). On the 9th day, 1mg of EB IM was administered (24 hours after the retreat of the PD4). The AIFT was done 54 hours after the removal of the PD4. It was used semen of three bulls distributed proportionally in each one of the 5 groups. The G-1 (n=31) group received a PD4 of second usage. The G-2 (n=32) group received a PD4 of third usage. The G-3 (n=33) group received a PD4 of third usage with two P4 new rings. The G-4 (n=28) group received a PD4 of fourth usage with two used rings and a new one. The G-5 (n=34) group received a PD4 of fourth usage with three new rings. The pregnancy diagnostic was done by ultrasound 30 days after the AIFT. The pregnancy rates (PR) were compared by chi-square test. Interaction among the treatments were not observed. The mean for groups G1, G2, G3, G4 and G5 were respctively: PR: 41.9% (13/31) vs. 43.7% (14/32) vs. 36.3% (12/33) vs. 39.2% (11/28) vs. 47.0% (16/34; $P>0.05$). According to resulted gotten in the present experiment, there are no significant differences among pregnancy rates in the analyzed groups. Therefore, we evidence that the fourth use of the DP4 (Cronipres®, Biogenesis) can be made, keeping pregnancy rates only when supplemented with P4 rings.

EFFECT OF TREATMENT WITH 0.5 mg ESTRADIOL BENZOATE GIVEN AT THE TIME OF CIDR-B INSERTION IN AN ESTRUS RESINCRONIZATION PROGRAM IN HEIFERS

Balla, E.[1,2]; Maraña Peña, D.[1,2]; Chesta, P.[2]; Pincinato, D.[1,2]; Peres, L.C.[1,2]; Tríbulo, R.[1,2]; Bó, G.A[1,2]

[1]Universidad Nacional de Córdoba, Argentina. [2]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

Two experiments were performed to evaluate the effect of treatment with 0.5 mg estradiol benzoate (EB) given at the time of CIDR-B insertion [1.9g progesterone, Pfizer Animal Health, Argentina] on return to estrus and pregnancy rates in heifers in an estrus resynchronization program. In Exp. 1, 121 Brangus and Braford heifers, 18 to 24 months of age and with a body condition score between 2.5 to 3.5 (1 to 5 scale) were used. All heifers were fixed time inseminated on Day 0 and 13 days later received a previously used CIDR-B and were randomly allocated to receive or not 0.5 mg de EB at the same time. CIDR-B devices were removed on Day 20 and heifers were observed for signs of estrus from Day 20 to 25 and inseminated (AI) 8 to 12 h after estrus. In Exp. 2, 187 heifers of the same age and breed from those in Exp. 1 were treated similarly except that CIDR-B devices were inserted on Day 14 and removed on Day 21. Pregnancy rates were determined by ultrasonography 28 days after the end of the AI period. Data was analyzed by logistic regression. There were no significant differences between groups on the distribution of return to estrus (P>0.5) [CIDR-B: Exp. 1: 54.4±5.0h; Exp 2: 72.0±5.1h and CIDR-B+0.5EB: Exp 1: 54.0±4.0h; Exp. 2: 75.5±4.8h]. Furthermore, no differences were detected on conception rates to the first AI [CIDR-B: Exp 1: 39/61 (63.9%); Exp 2: 46/94 (48.9%) and CIDR-B+0.5EB: Exp 1: 35/60 (58.3%); Exp 2: 49/93 (52.7%); P>0.1], return to estrus [CIDR-B: Exp 1: 15/22 (68.2%); Exp 2: 20/49 (40.8%) and CIDR-B+0.5EB: Exp 1: 18/25 (72.0%); Exp 2: 17/44 (38.6%); P>0.1], conception rate to the second AI [CIDR-B: Exp 1: 10/15 (66.7%); Exp 2: 7/20 (35.0%) and CIDR-B+0.5EB: Exp 1: 11/18 (61.1%); Exp 2: 10/17 (58.8%); P>0.1] and overall pregnancy rates [CIDR-B: Exp 1: 49/61 (80.3%); Exp 2: 53/94 (56.4%) and CIDR-B+0.5EB: Exp 1: 46/60 (76.7%); Exp 2: 59/93 (63.4%); P>0.1]. Results demonstrate that the use of 0.5 mg EB at the time of insertion of a CIDR-B in an estrus synchronization program, does not have an effect on the either the return to estrus and pregnancy rates.

EFFECT OF DOSAGE OF ESTRADIOL BENZOATE ON PREGNANCY RATES IN HEIFERS SYNCHRONIZED WITH CIDR-B DEVICES

Balla, E.[1,2]; Maraña Peña, D.[1,2]; Chesta, P.[2]; Pincinato, D.[1,2]; Peres, L.C.[1,2]; Tríbulo, R.[1,2]; Bó, G.A.[1,2]

[1]Universidad Nacional de Córdoba, Argentina. [2]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

Two experiments were designed to evaluate the effect of treatment with 1 or 2 mg of estradiol benzoate (EB) given at the time of insertion of a CIDR-B device (1.9 g progesterone, Pfizer Animal Health, Argentina) on pregnancy rates in heifers that were inseminated at a fixed-time (FTAI). In experiment 1, Brangus and Braford heifers (n=125), 20 to 26 months of age and with a body condition score between 2.5 to 3.5 (1 to 5 scale) were treated with a new CIDR-B device and randomly divided to receive 1 or 2 mg EB (Estradiol 10, Lab. Río de Janeiro, Argentina) at the same time (Day 0). All heifers received 500 μg cloprostenol (PGF) at the time of CIDR-B removal (Day 8), 1 mg EB 24 h later (Day 9) and were FTAI 28 to 32 h after EB (Day 10). In experiment 2, heifers of similar age and breed of those in experiment 1 (n=187) received (Day 0) 1 or 2 mg EB and were subdivided to receive a new CIDR-B device or a CIDR-B previously used twice (third-use CIDR-B) that was sterilized with autoclave. The third-use CIDR-B were washed and disinfected with a quaternary ammonium solution at 0.5% concentration (Bagodryl, San Jorge Bagó, Argentina) during 10 minutes and were then autoclaved (1.5 Atm., 122 ºC, during 20 minutes). CIDR-B devices were removed on Day 7, 1 mg EB was given on Day 8 and all heifers were FTAI on Day 9 (28 to 32 h after EB). In both experiments pregnancy rates were diagnosed by ultrasonography (100 Falco Vet., Pie Medical, with and 8 MHz transducer) 70 days after FTAI. Pregnancy rates were compared by chi-square test in experiment 1 and by Mantel Haenszel test in experiment 2. There were no significant differences (P>0.24) in pregnancy rates between groups in both experiments [1 mg EB, Exp. 1: 34/62 (54.8%) and Exp 2: 50/97 (51.5%); 2 mg EB, Exp 1: 41/63 (65.1%) and Exp. 2, 45/90 (50.0%)]. Furthermore, pregnancy rates did not differ (P>0.71) between heifers treated with new CIDR-B (46/93, 49.5%) or third-use CIDR-B (49/94, 52.1%). Results demonstrate that the administration of 1 or 2 mg EB at the time of insertion of a new or used CIDR-B did not affect pregnancy rates in Brangus and Braford heifers FTAI.

PREGNANCY RATES IN NELORE COWS AFTER TIMED ARTIFICIAL INSEMINATION AND SUPPLEMENTATION WITH LUTEOTROPHIC OR ANTI-LUTEOLYTIC AGENTS

Machado, R.[1]; Bergamaschi, M.A.C.M.[1]; Barbosa, R.T.[1]; Alencar, M.M.[1]; Madureira, E.H.[2]; Barbosa, P.F.[1]; Bisinotto, R.S.[2]; Binelli, M.[2]

[1]EMBRAPA Pecuária Sudeste, São Carlos-SP, C.P. 339, 13560-970, Brasil. [2]Departamento de Reprodução Animal - FMVZ/USP, Pirassununga-SP, 13635-900, Brasil. rsbisinotto@yahoo.com.br

Embryonic losses in beef cows can reach up to 40% and its majority takes place in early gestation due to failure of pregnancy recognition and consequent luteolysis. In the present study, different approaches were tested in order to reduce pregnancy losses after timed artificial insemination (TAI). Specifically, aims were to optimize luteal function through administration of hCG on the 5th day after TAI and/or inhibit follicular function through the administration of 17β-estradiol (E_2) on the 12th day after TAI. Two hundred and twenty non-suckled adult Nelore cows (*Bos taurus indicus*) with at least 240 days postpartum, had their ovulations synchronized ("OvSynch") and received TAI 16 hour after the 2nd GnRH. Animals were divided in four groups with 55 cows each: Control (Tc) did not receive anything else, hCG (T_{hCG}) received 3000 UI of hCG (i.m.) on D_5, E_2 (T_{E2}) received 5 mg of E_2 (i.m.) on D_{12} and group hCG/E_2 ($T_{hCG/E2}$) received 3000 UI of hCG (i.m.) on D_5 plus 5mg of E_2 (i.m.) on D_{12}. TAI was day "D_0" of the experiment. Between D_5 and D_{64} estrus behavior was monitored twice daily with the aid of two teaser bulls per group. Pregnancy rates at TAI (PR_{TAI}) and cumulative (PR_{cum} = PR_{TAI} plus AIs throughout the experiment) were determined. Pregnancy was measured on days D_{31} and D_{99} by transrectal ultrasonography. Rates were analyzed by Chi-square (χ^2) using the Proc FREQ of SAS. Between group differences in PR_{TAI} were detected (P<.05). The PR_{TAI} of Tc and T_{hCG} tended (.10>P>.05) to be higher (34,5% and 36,5%, respectively) than $T_{hCG/E2}$ (18.2%). In addition, PR_{TAI} of T_{E2} (5.4%) was lowest (P<.05). The PR_{cum} of cows receiving E_2 on D_{12} (T_{E2} + $T_{hCG/E2}$) was lower (66.4%; P<.05) than that observed to cows that did not receive (T_C + T_{hCG}) this estrogen (80.9%). The effects of hCG on PR_{TAI} and PR_{cum} were not significant (P>.05). In conclusion, luteotrophic supplementation with hCG given to non-suckled, cyclic Nelore adult cows did not increase pregnancy rates and rescheduling ovarian function with E_2 after TAI reduced pregnancy rates and such reduction was not reverted throughout the experiment.

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OVULATION RATE IN FEMALE BUFFALOES INSEMINATED WITH A FIXED TIME PROTOCOL

Stella, E. D.[1]; Oba, E.[1]; Mota, A.V.[1]; Lima Neto, J.F.[1]; Gioso, M.M.[1]

[1] Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ – Unesp. Distrito de Rubião Júnior S/N, 18618-000 – Botucatu – SP – Brasil – edaostella@fmvz.unesp.br

The objective of the present study was to evaluate the responses of three diferents hormonal protocols with CRESTAR® in buffaloes cows in the breeding season (February – March) aiming ovulation rate. Transrectal ultrasonography using a 5.0 MHz transducer probe (Aloka 500), were done daily in order to study follicular dinamics at each 12 hours after implant removal to verify the moment of ovulation. Twenty four cows with more than 45 days of post partum period were randomly allocated into three groups (Group 1=G1, n=8; Group 2=G2, n=8 and Group 3=G3, n=8). All buffaloes (G1, G2 and G3) received an auricular implant progesterone releasing device (3mg of norgestomet, CRESTAR®, Intervet) plus 2.0mg estradiol benzoate (IM; Benzoato de Estradiol®, Index) at random stages of estrous cycle (Day 0, PM). The implants remained for 9 days. CRESTAR® was removed and a luteolytic dose of PGF2α analog (0.15mg d-cloprostenol, Croniben®, Biogenesis) was administered on Day 9 (D9, PM). On this day, only buffaloes in G1 and G3 received 500 UI of eCG IM (Novormon®, Syntex). After two days (D11), buffaloes in groups 1 and 2 (G1 and G2) received 1000UI of hCG (Vetecor, Calier) 42 hours after implant removal. All animals were timed artificially inseminated 54 after PGF2α . The statistics analysis was made by Chi-square tests (X^2), ovulation rates in three groups (G1, G2 and G3) was 62,5, 50 and 75% respectively. There wasn´t found statisticals differences in ovulation rates between the groups until day 13 (P>0.05).

DO TEMPORARY CALF REMOVAL (TCR) AND/OR ECG ADMINISTRATION INCREASE PREGNANCY RATES IN LACTATING NELORE COWS TREATED WITH PROGESTERONE RELEASE INTRAVAGINAL DEVICE?

Pinheiro, V.G.[1a]; Souza, A.F.[1a]; Pegorer, M.F.[1]; Ereno, R.L.[2]; Barros, C.M.[1]

[1]Departamento de Farmacologia, Instituto de Biociências, [2]Departamento de Reprodução Animal, FMVZ-UNESP, Botucatu-SP, Brasil. viniciusgpinheiro@yahoo.com.br

There are reports in the literature that either TCR or eCG administration can increase the efficiency (pregnancy rate) of hormonal treatments with progesterone/progestagens during postpartum anestrus. In the present work association of TCR and/or eCG administration were tested simultaneously in a protocol with progesterone, frequently used for fixed-time artificial insemination (FTAI) in cows during postpartum anestrus. The protocols were tested in 2 properties, in which lactating Nelore cows (40 to 70 d post-partum, n=281), were allocated in 4 Groups: PEPE, PEPE/TCR, PEPE/eCG and PEPE/TCR/eCG. At random stage of the estrous cycle (D0), animals from Group PEPE (Progesterone-Estrogen-Prostaglandin-Estrogen) received an intravaginal device with progesterone (1.0 g, DIB®) and 2.5 mg of estradiol benzoate (EB, i.m., Estrogin®). Eight days later (D8) cows were treated with PGF2α (150 mcg d-cloprostenol, i.m., Prolise®), and DIB was removed. Twenty four hours after DIB removal, cows received EB (1.0 mg, i.m), and 30 to 36 h later all animals were FTAI, without estrus detection. In Group PEPE/TCR, cows received the same treatment described above (PEPE) and the calves were removed during 54 h (from DIB removal until FTAI). In Group PEPE/eCG, animals received PEPE treatment plus one dose of eCG (300 UI, i.m., Novormon®) following PGF2α administration (D8). In Group PEPE/TCR/eCG, animals were treated as in protocol PEPE/TCR plus eCG on D8. All animals were examined by ultrasonography (Aloka SSD 500, 7.5 MHZ probe) 10 days before and at the beginning of hormonal treatment, in order to detect anestrous cows (absence of CL in both exams). Pregnancy diagnosis was performance 30 days after FTAI, by ultrasonography. The data were analyzed by logistic regression. The following variables were considered by the model and did not interfere with pregnancy rates: farms, inseminators, and semen (sire). Eighty percent (80%) of the animals were in anestrus (absence of CL) and the pregnancy rates were similar ($p>0.05$) among the 4 groups: PEPE (27/62 = 43.6%), PEPE/TCR (29/73 = 39.7%), PEPE/eCG (31/71 = 43.7%), and PEPE/TCR/eCG (31/75 = 41.3%). The results indicate that in Nelore cows, in post-partum anestrus, and good body conditions, TCR and/or eCG administration do not improve the efficiency (pregnancy rate) of protocol PEPE. New experiments are underway to confirm the present findings in a larger number of animals.

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EFFECT OF THE POST PARTUM INTERVAL ON CONCEPTION RATES IN FIXED TIME ARTIFICIAL INSEMINATION USING CIDR IN NELORE SUCKLED COWS

Barrreiros, T.R.R.[1]; Baruselli, P.S.[2]; Balarin, O.F.[3]; Seneda, M.M.[4]

[1]Faculdade Integrado de Campo Mourão/PR[2], [2]Departamento de Reprodução Animal FMVZ – USP, [3]Apec Consultoria Agropecuária - Cornélio Procópio/PR, [4]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051- 990 thalesrigo@grupointegrado.br

The aim of this trial was to evaluate the post partum interval effect on conception rates on conception rates of Bos indicus cattle submitted to fixed time artificial insemination (FTAI). The work was made in farm located in Santa Rita do Pardo/MS. Two hundred and fifty five Nelore suckled cows, 2,5 to 4 body score condition and were divided into three groups related to post partum interval: G1 (n=79), G2 (n=73) e G3 (n=103), respectively with 40 to 49, 50 to 57 and 58 to 65 days. The animals received 1.9g implant of intravaginal progesterone (CIDR, Pfizer, Brazil) and 2 mg estradiol benzoate IM (Estrogin, Farmavet, Brazil), in random stages from estrous cycle. On eight day the implants were removed and the animals received an administration IM of 25 mg Dinaprost (Lutalyse, Pfizer, Brazil). After 24h all animals received 1 mg estradiol benzoate IM. All animals were inseminated 52-56h after implants removal. The pregnancy diagnosis was made by transrectal ultrasonography (Aloka SSD 500, 5.0 mHz) after 45 days from artificial insemination. Data was analyzed by Chi–Square test. There were no significant differences in pregnancy rates for G-1 (49.3% 39/79), G-2 (53.4% 39/73) and G-3 (56.3% 58/103); (P>0.05). There was no interaction between body score condition and conception rates. The results demonstrate that P4 based treatments for FTAI were effective to reduce the interval partum-conception for showed acceptable conception rates from 40 days post-partum. This is a viable alternative for increase reproductive efficiency in commercial *Bos indicus* herds.

ARTIFICIAL INSEMINATION AT FIXED TIME IN HIGH-PRODUCING HOLSTEIN COWS WITH ESTRADIOL BENZOATE AND CLOPROSTENOL

Silveira, E.C.[1]; Barrreiros, T.R.R.[2]; Delboni, M.I.[3]; Oliveira, A.C.[3]; Padilha, L.C.[3]; Seneda, M.M.[4]

[1]Produtiva Centro de Tecnologia Pecuária, Londrina/PR, [2] Faculdade Integrado de Campo Mourão/PR, [3]Alunas da graduação de Medicina Veterinária da Universidade Estadual de Londrina, [4]Departamento de Clínicas Veterinárias, CCA, UEL, PR, 86051- 990 thalesrigo@grupointegrado.br

The objective of this study was to evaluate the artificial insemination protocol at fixed time in dairy cattle. Four hundred and fourteen Holstein cows were used from 50 to 180 days post-partum, 24 to 96 months old and body condition score ranging from 2,5 to 3 (score 1 - 5). The experiment was conducted in two farms in Londrina and Rolândia, both in Paraná State, from May to December 2004. The animals were dived in two groups according to body condition score and post-partum period. The control group (G-CON, n=214), was observed early in the morning and late in the afternoon for estrus detection and then inseminated 12 hours later the detection. In the EPE group (G-EPE, n=200), the animals were injected randomly estradiol benzoate (Be); (Estrogin, Farmavet, Brazil) during estrous cycle intramuscular (IM). On the eighth day the animals received 150 μg of cloprostenol (Veteglan, Calier, Brazil) IM. After twenty-four hours, all cows received 1 mg of Be IM, and 30 hours later, the animals were inseminated at fixed time. Pregnancy was diagnosed by rectal palpation at 60 days after the artificial insemination. The results were analyzed in agreement with the test of the corrected Chi-square of Yates. In both properties, no significant differences were detected ($P>0.05$) when conception rates were grouped, and results were 37.3% (80/214) and 40.5% (81/200), respectively for G-CON and for G-EPE. Although conception rates have not been increased using this protocol, it was possible to observe acceptable results, as well as the low cost of this treatment, which can help its wider use.

EFFICIENCY OF REDUCED DOSES OF FERTIRELIN ACETATE IN OVSYNCH PROGRAMS IN DAIRY COWS

Gioso, M.M.[1]; Fernandes, C.A.C.[1,2]; Oba, E.[1]; Carvalho, M.A.P.[3]; Vasconcelos, T.D.[2]

[1] Department of Animal Reproduction and Veterinary Radiology - FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. [2] Biotran LTDA – Alfenas- MG, Brazil. [3] Medicate Veterinarian.
mmgioso@yahoo.com.br

Many similar products to GnRH are effective in protocols of fixed time artificial insemination (FTAI). However, Acetate of Fertirelin is a analog of this hormone whose information about action in the bovine follicular dynamics is inconsistent. Therefore, the objective of this work was to compare the efficiency of two doses different from Fertirelin, for synchronization of follicular wave emergence and induction of the ovulation, in dairy cows submitted to Ovsynch protocol. Were used seventy seven dairy cows, on 60 to 120 days postpartum and body score of 2.5 to 4.0 (range 1 to 5). The animals were previously submitted to ultrasound evaluation using a linear transducer (6/8MHz, ESAOTE, model Falco-100). Animals presenting a reasonable number of follicles either witch a presence of corpus luteum (CL) or without CL but witch 10 mm follicles were introduced in the experiment. The females were randomly distributed in four treatments: Group 1 (n=21) - 100 μg (full dose) of Fertirelin (Fertigen®, Schering Plough - Coopers, Brazil) at day 0, PGF2α (500 μg; Ciosin®, Schering Plough - Coopers, Brazil) at day 8 and 100 μg of Fertirelin at day 10. Group 2 (n=21) - 100 (g of Fertirelin at day 0, PGF2α (500 (g) at day 8 and 50 (g of Fertirelin at day 10. Group 3 (n=19) - 50 (g of Fertirelin at day 0, PGF2α at day 8 and 100 (g of Fertirelin at day 10. Group 4 (n=16) - 50 (g of Fertirelin at day 0, PGF2α (500 (g) at day 8 and 50 (g of Fertirelin at day 10. The administrations of drugs were accomplished with needles 25X07, through intramuscular route. Evaluations of ultrasound were accomplished during the treatments and the images were recorded in VHS to measure of the follicles and CLs. Follicular wave induction rates after first Fertirelin administration and induction of ovulation rates after second administration of the same product were compared by Qui-square test. The effects of the treatments in the different days of analysis about the values of the diameter of ovulatory follicle at day of PGF2α administration and at day of Fertigen® administration were submitted by ANOVA. The mean values of dominant follicles in the groups were similar at day 8 (group 1: 1.51cm; group 2: 1.51cm; group 3: 1.56cm and group 4: 1.54cm) and at day 10 (group 1: 1.55cm; group 2: 1.62cm; group 3: 1.59cm and group 4: 1.82cm; p>0.05). The synchronization rates of the first follicular wave in the four treatments were similar: 50.0%; 78.5%; 76.9% and 75.0%, respectively (P>0.05) with mean of 70.0%. The ovulation rates at the end of treatments were similar in the four groups: 66.6%; 52.3%; 63.2% and 87.5%, respectively (P>0.05) with mean of 66.2%. The effects reached by 50 μg and 100 μg doses of Fertirelin in the protocols of FTAI are similar to found by other products mentioned in the literature (Bartolome et al., J. Dairy Sci., v.85, p.99, 2002). In conclusion, Fertirelin Acetate was efficient inducing emergency of a new follicular wave, as well as the induction of the ovulation at the end of the protocol, using the dose of either 50μ or 100 μg, and it is suggested that dose of 50μg of Fertirelin can be recommended without compromising in the final results of the program.

COMPARITIVE STUDY OF TWO PROTOCOLS FOR SYNCHRONIZATION OF OVULATION AND FIXED-TIME INSEMINATION IN COWS

Abreu, C.O.[1]; Martinez, A.C [1]; Pires, R. M. L.[2]; Centenaro, E.A.[3]; Bertonsello, L.G.[3]; Zampiva, W.K.[3]

[1]Animal reproduction post graduated student, FCAV – UNESP, Jaboticabal. cassianabreu@ig.com.br [2] Scientific researcher of, APTA - Instituto de Zootecnia de Nova Odessa [3] Veterinary graduated student of Paraná State University.

The artificial insemination (AI) needs the estrus observation to be efficient, and it's a disadvantage for its application. The use of hormones to induce ovulation allows the AI with fixed time, with doesn't needs the estrus observation. The goal of this study was compare the Presynch and Ovsynch protocols to induce the ovulation and AI with fixed time. It was used 17 Nelore cows in each group. In the Ovsynch treatment, was administrated 200 μg of Gonadorelin (Profertil® - Tortuga Companhia Zootécnica, São Paulo, Brasil) in beginning of treatment (D0). At D7 were applied 150μg of D+ Cloprostenol (Prostaglandina Tortuga®) and in D9 were applied 200·μg de Gonadorelina (Profertil®). All animals were inseminated between 20 to 24 hours after the last application. In the Presynch treatment, were administrated in D0 150μg of D+ Cloprostenol (Prostaglandina Tortuga®). At D14 were applied the same hormone in the same dose, and two days after were administrated 200 μg de Gonadorelina (Profertil®). All animals were inseminated two days after, between 20 to 24 hours after the last application. The estrus was observed for twice a day, for 45 minutes each time. The Presynch protocol was as efficient as the Ovsynch (58.82% and 47.06% of non-return rate, respectively, $p>0.05$, in Qui square test), but was US$ 5.00 cheaper per cow than Ovsynch.

FOLLICULAR DYNAMICS OF NELORE COWS TREATED WITH ESTRADIOL CYPIONATE OR BENZOATE IN FIXED TIME ARTIFICIAL INSEMINATION PROTOCOLS

Martins, C.M.[1]; Castricini, E.S.C.[1]; Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ – USP, São Paulo – SP, 05508-000, Brasil.
barusell@usp.br

The effects of ovulation inducer [Estradiol cypionate (EC) or Estradiol benzoate (EB)] or the moment of its administration (day of intravaginal progesterone releasing device removal or 24 hours later) in follicular dynamics in Nelore cows (*Bos indicus*) were evaluated. Thirty three suckling Nelore cows (n=33) were homogeneously assigned in four groups (2x2 factorial) according to body score condition and postpartum period. On random day of estrous cycle (Day 0), the animals received 2mg of EB, i.m. (Estrogin®, Farmavet, Brasil) and a intravaginal progesterone releasing device (CIDR®, Pfizer, Nova Zelândia). On Day 8, the devices were removed and PGF2α i.m. (Lutalyse®, Pfizer) was administered. Groups EC0 (n=9) and EC24 (n=8) were treated with 0,5mg of EC (ECP®, Pfizer) at device withdrawal or 24 h later, respectively. In Groups EB0 (n=8) and EB24 (n=8) 1mg of EB was administered at the same moment than other groups (EC). Ovulatory rate (OVR), moment of ovulation (OVM) and maximum diameter of dominant follicle (MDDF) were determined by ultrasonographic examination each 12h. Data were analyzed by Anova, Chi-square and Bartlett tests. No interaction was observed between treatment and moment of inducer administration. The main effects to EC vs. EB were, respectively: OVR [88.0% (15/17) vs. 81.0% (13/16); P>0.05]; OVM ($76.8\pm2.82^{a.x}$ vs. $67.4\pm1.69^{b.y}$ hours; P<0.05), MDDF (1.28±0.04 vs. 1.35±0.03 cm; P>0.05). The effects of moment of inducer administration (0 vs 24 h) were: OVR [94.0% (16/17) vs. 75.0% (12/16); p>0.05], OVM (68.3 ± 1.81^{a} vs. 78.0 ± 3.01^{b} hours; p<0.05), MDDF (1.30±0.02 vs. 1.34±0.05 cm; P>0.05). Results suggest that estradiol cypionate administration delays and promotes the ovulation widespread. Also, the injection of both inducers (estradiol cypionate or benzoate) at moment of intravaginal progesterone releasing device withdrawal reduces the interval between device removal to ovulation in Nelore cows.

EMBRYO PRODUCTION IN HOLSTEIN COWS AT DIFFERENT PROTOCOLS OF SUPERSTIMULATION WITH FIXED TIME ARTIFICIAL INSEMINATION

Martins, C.M.[1]; Castricini, E.S.C.[1]; Reis, E.L.[1]; Torres-Júnior, J.R.S.[1]; Gimenes, L.U.[1]; Sá Filho, M.F.[1]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo/ SP, 05508-000, Brasil.
barusell@usp.br

A total of 16 Holstein cows (>30L/day and ≥60 milking days) housed in tie stall were assigned in four groups: P24LH48; P24LH60; P36LH48 e P36LH60 (2x2 factorial; cross-over). The animals received a intravaginal progesterone releasing device (P_4; DIB®, Syntex, Argentina) on Day -1 and 2mg of estradiol benzoate (BE; Estrogin®, Farmavet, Brazil) on Day 0. Superstimulation was performed with 200mg of FSHp (Folltropin-V®, Bioniche, Canada) in 8 decreasing doses each 12 hours, from Day 4. On Day 6, PGF2α (Prolise®, Tecnopec, Brazil) was administered. Related to PGF2α, P_4 devices were removed 24 (**P24)** or 36 (**P36**) hours later and LH (25mg de LH, Lutropin-V®, Bioniche, Canada) 48 (**LH48)** or 60 (**LH60)** hours later. Inseminations were performed 12 and 24 hours after LH using the same ejaculate of only one sire. Embryo recovery was performed on Day 15. The data were analyzed by Anova and Chi-square tests. No interaction was observed between the variables. The main effects for treatments P24 vs P36 and LH48 vs LH60 were, respectively: ovulation rate (49.9±5.7 vs 60.9±4.8% and 53.1±5.3 vs 57.5±5.4%; $p>0.05$), recovery rate (67.4±5.8 vs 69.9±6.4% and 64.4±6.5 vs 73.1±5.5%; $p>0.05$), total of structures (4.4±0.9 vs 5.0±0.9 and 3.8±0.7 vs 5.7±1.0; $p>0.05$), Grade 1 embryos (2.4±0.6 vs 3.2±0.7 and $1.6±0.4^a$ vs $4.0±0.8^b$; $p<0.05$), degenerate embryos (0.9±0.2 vs 0.6±0.3 and 1.0±0.3 vs 0.5±0.1; $p>0.05$), transferable embryos (3.0±0.7 vs 4.1±0.9 and $2.3±0.5^a$ vs $4.9±0.9^b$; $p<0.05$) and suitable to freezing embryos (2.9±0.7 vs 3.8±0.8 and $2.1±0.5^a$ vs $4.7±0.9^b$; $p<0.05$). There was effect of LH administration moment (LH60) on number of Grade 1, transferable and suitable to freezing embryos. Results suggest that administration of LH 60 hours after PGF2α increases the embryo production in high production Holstein cows superstimulated and artificially inseminated at fixed time.

(Acknowledgments: **Tecnopec**)

EFFECT OF PGF2α AT INSERTION AND OF eCG AT REMOVAL OF INTRAVAGINAL PROGESTERONE RELEASING IN PREGNANCY RATES TO FIXED-TIME ARTIFICIAL INSEMINATION IN NELORE HEIFERS

Marques, M.O.[1]; Sá Filho, M.F.[1]; Gimenes, L.U.[1]; Figueiredo, T.B.[1]; Sória, G.F.[2]; Baruselli, P.S.[1]

[1]Departamento de Reprodução Animal, FMVZ/USP, São Paulo-SP [2]Departamento de Reprodução, UNESP, Jaboticabal-SP. barusell@usp.br

The aim was to evaluate the effect of prostaglandin (PGF2α) administration at the insertion and/or equine corionic gonadotrofin (eCG) at removal of intravaginal progesterone releasing device (CIDR-B) in Nelore heifers *(Bos indicus*) submitted to fixed-time artificial insemination (FTAI). A total of 392 heifers (>300Kg, kept on pasture) were evaluated by ultrasonography and classified into three groups according to their ovarian status [A- with corpora luteal (CL); B- without CL but with dominant follicle (FD) ≥ 8mm or C- without CL and with FD <8mm]. At the beginning of the treatment (Day 0) all heifers received a CIDR (Pfizer, Brazil) plus 2mg of estradiol benzoate i.m. (EB; Estrogin®, Farmavet, Brazil). On Day 8, the devices were withdrawn and a PGF2α i.m. (Lutalyse®, Pfizer, Brazil) was administered. On Day 9, 1 mg of EB i.m. were administered and the heifers were submitted to FTAI 30-34 hours later. The semen utilized has proven fertility and the ejaculates were homogeneously assigned among the experimental groups. The Control Group did not receive additional treatment, while the PGF group received a PGF2α dose on Day 0, the eCG Group received 400 IU of eCG (Novormon®, Syntex, Argentina) on Day 8. The PGF/eCG Group received both treatments. The pregnancy diagnosis was performed by ultrasonography 30 days after insemination. The data were analyzed by Chi-square test. No interaction was observed among the treatments. The effect of PGF2α (without vs. with PGF2α), eCG (without vs. with eCG) treatments and ovarian status in the pregnancy rates were: without PGF2α (24,7%; 49/198) vs. with PGF2α (25,8%; 50/194), without eCG (15,7%[a]; 31/197) vs. with eCG (34,9%[b]; 68/195) and ovarian status A. (27,6%; 63/264), B (20,6%; 20/97) e C (19,4%; 6/31). In conclusion the eCG treatment increased the pregnancy rate. The PGF2α administration did not improve conception rate in Nelore heifers.

Acknowledgments: Pfizer

SUPERSTIMULATION WITH FIXED-TIME ARTIFICIAL INSEMINATION IN *Bos indicus* DONORS TREATED WITH NORGESTOMET AURICULAR IMPLANT OR PROGESTERONE INTRAVAGINAL RELEASING DEVICE

Chiari, J.R.[1]; Demétrio, D.[1]; Sá Filho, M.F.[2]; Ayres, H.[2]; Baruselli, P.S.[2]

[1]SAMVET, Morrinhos-GO, Brasil. [2]Departamento de Reprodução Animal, FMVZ/ USP, São Paulo/ SP, Brasil. barusell@usp.br

The aim of this study was to evaluate Norgestomet auricular implant (Crestar) or progesterone intravaginal releasing device (CIDR) in superstimulation with fixed-time artificial insemination (SOFT) protocols in Guzera donors *(Bos indicus).* A total of 30 females was homogeneously assigned in two experimental groups (Crestar or CIDR). In the beginning of treatment (Day 0), the females of Crestar Group (n=15) received a Norgestomet auricular implant (Crestar, Intervet, Brazil), while that of CIDR Group (n=15) received a progesterone intravaginal releasing device (P4; CIDR, Pfzier, Brazil). In both groups 2,0 mg of estradiol benzoate were associated to 50mg of P4 i.m.(Index Farmaceutica, Brasil). The superstimulatoy response was performed by 133mg of FSHp (Folltropin, Bioniche, Canadá) administration in 8 descending doses at 12 hours interval from Day 4. On Day 6, two PGF2α (Preloban, Intervet, Brasil) doses (at morning and at afternoon) were administered. The implant/ device was withdraw 36 hours (P36) and 250mg of GnRH (Fertagil, Intervet, Brasil) were administered 48 hours after the first PGF2α dose. The response to treatments was evaluated by ovarian ultrasonography. Data were analyzed by Anova and Chi-square tests. The results of Crestar vs. CIDR Group were, respectively: follicles >8mm at embryo collection (1.2±0.4 vs 1.3±0.3; p>0,05), *corpus luteum* at embryo collection day (7.0±1.0 vs. 6.5±1.0; p>0.05), recovery rate (89.3±5.1 vs. 83.6±6.0%; p>0.05), total of structures (6.0±0.9 vs 5.3±1.0; p>0.05), transferable embryos (3.1± 1,0 vs. 3.3±0.9; p>0.05), unfertilized structures (1.7±0.6 vs 0.50±0.3; p>0.05) and degenerated structures (1.1±0.2 vs. 1.3±0.4; p>0.05). No differences among Crestrar and CIDR Groups were observed in analyzed variables. Results are suggestive that Norgestomet auricular implants and P4 intravaginal releasing devices presents similar response in SOFT protocols.

Acknowledgements: Intervet

THE INFLUENCE OF HORMONAL DOSES ON THE CONCEPTION RATE IN ZEBU COWS SUBMITTED TO PROTOCOLS OF SYNCHRONIZED OVULATION FOR FIXED TIME INSEMINATION

Ribeiro Filho, A. de L.[1]; Bittencourt, R.F.[1]; Chalhoub, M.[1]; Alves, S.G.G.[1]; Biscarde, C.E.A.[1]; Almeida, A.K.[1]; Portela, A.P.M.[1]; Freitas, D.S.[1]; Silva, A.A.B.[1]; Santana, R.C.M.[1];

[1]Escola de Medicina Veterinária – UFBA, Salvador-BA, 40170-110, Brasil. alisboa@ufba.br

A group of two hundred zebu cows was randomly split in tree experimental groups. Before the experience started, the following aspects were evaluated: age, corporal score rate and reproductive status of each animal. The aim of this study was to evaluate the influence of hormonal doses on the conception rate of zebu cows submitted to protocols of synchronized ovulation for fixed time insemination. In the group 1 (G1 – n=102), the animals received 3mg de Norgestomet (NOR) through auricular implant, on random stage of oestrous cycle on the Day 0. Right after it, an IM injection of 3mg of oestradiol valerate (OV) plus NOR (5mg) was administered. In the Group 2 (G2 – n=20) and Group 3 (G3 – n=78) animals, the same protocol was used, although, right after the auricular implant application, in the G2, it was administered 4.5mg of NOR and 7.5mg of OV. In the G3, the auricular implant was used again (for the second time) and the IM administered concentrations were similar to G2's. Nine days after (D9), the implants were removed and the animals were inseminated on a fixed time (FTI), approximately 56h after the implant removal (D11). The inseminations were made by the same inseminator and the semen of only one bull was used. The semen samples were evaluated using only those who reached the basic requirements regarding physical and morphological characteristics. The pregnancy diagnosis, performed 60 days after the FTI, didn't present differences ($P>0.05$) when evaluated by the qui-square (χ^2). The pregnancy rates were 33%, 19% and 41%, respectively, for G1, G2 and G3. Also the corporal score didn't present differences ($P>0.05$), among the groups. Thus, it shows the possibility of the auricular implant reutilization (twice), without compromising the pregnancy rates of zebu females submitted to the synchronization protocols for FIT.

THE INFLUENCE OF CORPORAL SCORE ON THE ZEBU HEIFERS, SUBMITTED TO A PROTOCOL OF SYNCHRONIZED OVULATION FOR FIXED TIME INSEMINATION

Bittencourt, R.F.[1]; Ribeiro Filho, A. de L.[1]; Chalhoub, M.[1]; Alves, S.G.G.[1]; Biscarde, C.E.A.[1]; Almeida, A.K.[1]; Portela, A.P.M.[1]; Freitas, D.S.[1]; Silva, A.A.B.[1]; Santana, R.C.M.[1];

[1]Escola de Medicina Veterinária – UFBA, Salvador-BA, 40170-110, Brasil. rfbvet@yahoo.com.br

A group of fifty nuliparous female was split in two experimental groups, aiming the study of body condition score (BCS) influence on the pregnancy rate of zebu heifers submitted to a protocol of synchronized ovulation for fixed time insemination (FTI). Before starting the experiment, all the animals were evaluated as for BCS, where the classification scale varied from 1 to 5: the females from 1 to 2 were classified as too thin; 2.5 as thin; 3 as moderate; 3.5 as good; 4 as fat and from 4.5 to 5 as too fat. Based on this classification, two experimental groups were taken. The animals with BCS lower than 3 were part of the first group (G1 – N=30), and the second group (G2 – N=20) was formed with the heifers with BCS equal or higher than 3. All the animals received 3mg of Norgestomet (NOR) through auricular implant, in random stage of oestrous cycle on the Day 0 (D0). Right after it, an IM injection of 2mg of oestradiol benzoate (OB) plus 50mg of progesterone (P4) was administered. Nine days later (D9), the implants were removed. On Day 10 the animals received 1mg of OB and were inseminated on fixed time (FTI), approximately 56h after the implant removal (D11). The inseminations were made by the same inseminator and the semen of only one bull was used. The semen samples were evaluated using only those who reached the basic requirements regarding physical and morphological characteristics. The pregnancy diagnosis, performed 60 days after FTI, presented differences among the studied groups ($P<0.02$), when evaluated by the qui-square (χ^2). Twenty two percent of the heifers got pregnant (11/50). The G1 and G2 animals' pregnancy rate were, respectively, 10% (3/30) and 40% (8/20). The zebu heifers corporal score interference, in the beginning of the oestrous cycle synchronization protocol for FTI demonstrated in this study, stands out its importance for the implementation of the conception final rates.

USE OF ULTRASOUND TO EVALUATE COWS IN FIXED TIME ARTIFICIAL INSEMINATION (FTAI).

Nogueira, E.[1,2]; Silva, A.S.[2]; Araújo, J.M.[2]; Tolentino, R.L.L.[3]

[1]Doutorando FCAV-UNESP eriklis@yahoo.com, [2]Gênesis Reprodução Animal, [3]Méd Veterinario

An experiment was carried to evaluate the use of ultrasound to verify the ovarian status of lactating cows and the influence in pregnancy rate in timed artificial insemination program. Two-hundred fifty two lactating Nelore cows were used in this study, with 71.3 days postpartum, body condition score: 5.2, in a scale of 1 to 9, and average weight 416.7 kg, in a farm in Mato Grosso do Sul. The synchronization protocol used was: a Progesterone Controlled intern Device release was maintained for 8 days, and 2 mg of Estradiol Benzoate injected at insertion of CIDR. At the Removal time 2 ml of PGF2a was injected, followed by an application of Estradiol Benzoate1 mg 24 hours later. The artificial insemination was performed 32 hours after the second application of EB. The ovarian status evaluation was performed at the CIDR insertion using a Falcon 100 (Pie Medical), with 8 MHz linear probe, and the animals were classified in three groups: CL- Corpus luteus present in one of the ovaries; F1: At least one follicle larger than 8 mm in the ovaries; F2: Follicles smaller than 8 mm in the ovaries. The data analysis, using ?2 test, showed the animal distribution in the groups was: 24.6; 49.2 and 26.2, for CL, F1 and F2, respectively, and the pregnancy rates at the first AI were: 56.7; 59.1; and 45.3, for these groups, higher ($p<0.05$) for groups CL and F1 than F2. At the second AI pregnancy rates there was no difference between the groups, with average values of: 53.5; 52.3; 50.1 for groups CL, F1 and F2, respectively. The data suggest that previous evaluation of postpartum ovarian status can be useful to elevate the pregnancy rates on FTAI programs, as we use postpartum animals with cyclicity, or even using hormones, like PMSG, or GnRH, in animals that don't have good cycllicity condition at the beginning of FTAI programs.

LASER ARTIFICIAL VISION SYSTEM: A TOOL FOR EVALUATION OF SEMEN UTILIZED IN REPRODUCTIVE BIOTECHNIQUES*

Carvalho, P.H.A.[1]; Barreto Filho, J.B.[1]; Rabelo, G.F.[2]; Braga Jr., R.A.[2]; Albuquerque, F.T.[1]; Rossi, R.O.S.D.[1]

[1]Departamento de Medicina Veterinária, [2]Departamento de Engenharia - UFLA, Lavras – MG, 37200-000, Brasil. barreto@ufla.br *Patente INPI – PI 0301926 - 8

The evaluation of spermatozoa viability after thawing is essential to the semen quality control, specially with the increase of biotechnologies such as *in vitro* embryo production. The evaluation by light and phase contrast microscopy is a simple and fast method for verification of the physical characteristics of semen. But, ranges of 30 to 60% have been reported when the same sample was submitted to the evaluation by different researchers (Iguer-Ouada & Verstagem, Theriogenology, v. 55, n. 3, p. 733-749, 2001). Use of a Laser Artificial Vision System (LAVS), which utilizes the *biospeckle* phenomenon, generates a value named the Inertia Momentum (MI), capable of quantifying the biological activity of illuminated material (Arizaga, Optics & laser technology, v. 4-5, p. 1-7, 1999), being able to come to be an objective method and which maintains semen viability, allowing its utilization in artificial reproduction processes. This work aimed to evaluate the quality of frozen semen of the bovine species by the SVAL, by comparing the MI generated by *biospeckle* with the vigor and motility parameters obtained in microscopy, total spermatic concentration and concentration of mobile cells, establishing correlations between the two methods. Thirty straws of 0.5 ml were thawed at 37º C for 30 seconds and aliquots of 10 µl were classified by microscopy as regards vigor and motility. Immediately, each sample was illuminated by the laser, generating successive images of the *biospeckle*, which were picked up by a camera and transformed into a space by time matrix, named the *Spatial Temporal Speckle*, from which MI was extracted. From each straw, an aliquot was taken, conserving it in formaldehyde-saline for the calculation of the total spermatic concentration and concentration of mobile cells. Spearman correlation was performed between the parameters evaluated by microscopy and the MI generated by the laser for each illuminated sample, by utilizing the SAS statistic program (SAS INSTITUTE SAS/STAT, SAS/IML software, version 6, 1990, 501p). The MI values presented a significant positive correlation ($p < 0.0001$) with the parameters of vigor ($r = 0.90$), motility ($r = 0.98$) and concentration of mobile cells ($r = 0.82$), showing consistency between biologic activity of semen and the index generated by LAVS. Between total spermatic concentration and MI there was no significant correlation ($p > 0.05$), showing there being not any significant influence of the concentration on the values of MI, for the spermatic concentrations found, which ranged from 10 to 500 X 10^6 spermatozoa per millimeter of semen. The results show that LAVS presents both sensitivity and consistency in evaluating the quality of post-thawing bovine semen, pointing to its possible use in the analysis of semen samples in animal reproduction biotechnology centers.

STANDARDIZATION OF A NEW HIPOSMOTIC TEST (HOST) FOR DOG SPERM EVALUATION.

Silva, K.V.G.C.[1,2]; Cunha, I.C.N.[2]; Rocha, A.A.[2]; Lopes, B.V.[2]; Silva, J.F.S.[2]

[1]-Experiment conducted during under graduation course of the author with PIBIC/CNPq scholarship and FAPERJ financial support. [2]-Laboratório de Melhoramento Genético Animal/CCTA/UENF- Av. Alberto Lamego, 2000. Campos dos Goytacazes - RJ. cunhaicn@uenf.br

Spermatozoa (sptz) with intact plasmatic membrane when exposed to hyposmotic condition struggle to equilibrate its osmotic pressure by absorbing water from the extra cellular compartment; as consequence an increase in intracellular volume will occur promoting the curly of the spermatic tail. In order to use this physiological principle of osmotic equilibrium as an indicative of sperm integrity the hyposmotic test (HOST) were developed (KUMI- DIAKA, Theriogenology, v.39, p.1279-89, 1993). According to BRITO *et al*. (Theriogenology, n.60, p.1539-51, 2003) HOST was a sperm evaluation method that significantly contributes in predicting *in vitro* fertilization rate. The hyposmotic solutions used in HOST ranges from 50 to 150 mOSM/L, although the use of distilled water as hyposmotic solution was also described (LIN *et al*., Fertility and Sterility, v.70, n.6, p.1148-55, 1998). The aim of the present study is to verify the possibility of using tap water as hyposmotic solution in HOST for canine sperm evaluation observing possible correlations between the HOST reactivity and progressive motility. Ten sperm samples from 4 distinct dogs with progressive motility ≥ 60% were extended up to 5ml in Kenney's extender and divided. One sub sample was three consecutive times submerged in N_2 in order to obtain a sample with knowingly damaged spermatic membrane (SL). The other sub sample with knowingly intact sptz (SI) was maintained in water bath at 37°C. Dilutions between the two sub samples (SL and SI) were performed in the following proportions: G1 – 100% of SL sample; G2 - 75% of SL sample and 25% of SI sample; G3 - 50% of SL sample and 50% of SI sample; G4 - 25% of SL sample and 75% of SI sample; G5 -100% of SI sample. After dilutions, all samples were again evaluated for progressive motility. Mean values for progressive motility (%) were: G1- 0±0; G2 – 24.5±13.01; G3 – 52±10.33; G4 - 67±9.2 and G5 – 82±11.1. One sample from each group was incubated in tap water from the lab sink (1:4 v/v) for five minutes in water bath (37 °C). The HOST result was evaluated under bright-field microscopy. For each group, 200 sptz were evaluated. Sptzs were considered intact (reactive (RE) to the HOST) when presenting curly tail. The mean values for RE sptz expressed in percentage (%) were: G1- 0.75±1.3; G2- 12.95±6.3; G3- 27±7.2; G4- 37.15±12.7 and G5- 50.85±17.6. High positive statistical correlation between the presence RE sptz and the knowingly intact sptz at each group ($R^2 = 0.9982$) and between the presence of RE sptz and progressive motility ($R^2 = 0.9843$) were observed. The results of the present study strongly suggest the potential use of tap water as hyposmotic solution to evaluate the integrity of dog spermatic membranes.

ANLYSIS AND CARACTERIZATION OF THE PROTEINS PROFILE OF SEMINAL PLASMA AND SPERM CELLS OF STALLION

Araújo, E.B.[1]; Souza, G.V.[1]; Silva, J.F.S.[1]; Fagundes, B.[1]

[1] Animal Genetic Improvement Department, UENF, Campos dos Goytacazes - RJ, Brazil
araujoeb@yahoo.com.br

The objective of this work was to characterize the proteins profile of seminal plasma (PS), sperm cells (CE) and both (PS+CE) before and after the concentration process of the proteins of these treatments, seeking to evaluate if the process of concentration of the proteins is efficient and to identify which treatment counts a larger final concentration of proteins. Besides, with the characterization of the proteins profile of the treatments we also aimed to evaluate if a variation or a repetition in the proteins profile between stallions, ejaculated and the different used treatments exist. In this experiment, it was used 3 stallions and it was collected 3 ejaculates from each, than it was collected three ejaculated that were processed and later their proteins were quantified and qualified for the methods of Bradford (Bradford, 1976) and for electrophoresis (Laemmlli, 1970). The average of proteins concentrations were 6,45mg/mL of PS+CE non concentrated, 47,20mg/mL of PS+CE concentrated, 0,58mg/mL of CE non concentrated, 4,06mg/mL of CE concentrated, 10,41mg/mL of PS non concentrated and 34,67mg/mL of PS concentrated showing that the process of protein concentration was efficient. Starting from the analysis of the profile of proteins found in the treatments of the three ejaculated collected of the three stallions we can verify that a variation exists from 9 to 26 proteic bands in the samples of PS, from 6 to 25 bands in the PS + CE and from 6 to 26 bands in the samples of proteins of CE. The electrophoretic profile of the samples showed a high variation of proteins between stallions and also between ejaculated of the same stallion. However, of all the evaluated proteins those that present molecular mass around 20 the 23 kDa had been the only ones that the treatments had been happened again in all this if must, very probably, to the fact of these proteins to be related to the process of reversion of the thermal shock (Barrios, 2000). In conclusion, the average concentrations of the proteins demonstrated that the process of concentration of the same ones in the filter AMICON® was efficient, the seminal plasma process of with sperm cells presented after concentration very hight final concentration of proteins, a great variation exists in the proteins profile between ejaculated, stallions and the different treatments and the proteins with molecular weight around 20-23 kDa were present in all samples.

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EFFECT OF TEMPERATURE ON MOTILITY DURING SEMEN COLLECTION AND COOLING PERIOD OF EQUINE SEMEN

Andrade, M.P.[1]; Silva, J.F.S.[1,3]; Souza, G.V.[1]; Fagundes, B.[1]; Costa, D.S.[1]; Detmann, E.[2]

[1] Animal Genetic Improvement Department, UENF, Campos dos Goytacazes-RJ, 28013-602, Brazil.
[2] Statistic Analysis Department, UFV, Viçosa-MG, Brazil

The stallion semen cooling for its transportation and subsequent artificial insemination has been enormously diffused in the equine industry. The aim of the present work is to optimize the conservation of fresh equine semen (6 ± 1ºC), increasing its survival through an immediate cooling at 22 ± 1ºC temperature in the moment of collecting and along its processing. The present work took place in Campos dos Goytacazes, Rio de Janeiro, Brasil, located in the Latitude 2145 South/Longitude 4117 West and Altitude 11m coordinates. Forty-two ejaculates from 3 half-blooded Mangalarga-Marchador stallions were used in this study. The experiment was carried out according to a totally randomized delineation, followed by a sub-divided parcels model, with the stallions being considered the main parcels and the ejaculates the sub-parcels. However, no source of interest was applied over the main parcels or the sub-parcels, and the "collection temperature" factor was given to the sub-parcels. The results were submitted to a variance analyses employing the type III square additions ($\alpha = 0.05$). Ejaculates were collected using a Hannover model artificial vagina (A.V.) modified by a device in an inverted "Y" shape, from the edges which flowed into two cups, one at room temperature and the other cooled at 22 ± 1ºC.The semen portion collected into the cooled cup showed better motility than the semen collected into the cup at room temperature, also after the cooled semen (6 ±1ºC) processing and at all subsequent moments of analysis (24, 48 and 72 hours). According to our results, the collection of equine semen using an artificial vagina equipped with a collector cup cooled at 22 ± 1ºC temperature is recommended.

Keywords*:* Equine, Semen, Cooled collect, Spermatic motility.

SEMINAL CHARACTERISTICS AND COOLING RESISTANCE OF CRIOULO BREED STALLION SPERMATOZOA – A PRELIMINARY STUDY

Boff, A.L.N.[1]; Curcio, B.R.[2]; Frey, F.J.[1]; Nogueira, C.E.W.[1]; Torres, A.[1]; Moraes, C.M.[2]; Bongalhardo, D.[3]

[1]Department of Veterinary Clinical – FV - UFPel; [2] Biotechnology Center-UFPel; [3] Department of Physiology and Pharmacology – IB - UFPel, Campus Universitário s/n 96010-900, Pelotas-RS. alnboff@yahoo.com.br

There are few records of seminal parameters of Crioulo breed stallions, and none of them establish the relationship between seminal characteristics and age. The objective of this study were 1) to document qualitative and quantitative seminal characteristics of young and adult Crioulo stallions; 2) to measure the effects of Kenney extender containing amicacin (KA) or gentamicin (KG) on the cooling of Crioulo stallion spermatozoa; and 3) to compare the effects of cooling plasma-free sperm with KA and KG. Semen of six stallions, three young (3 years-old) and three adults (>8 years-old) was collected using artificial vagina (August-October/2004). Qualitative (motility, vigor, morphology) and quantitative (volume and concentration) seminal characteristics were evaluated. Each ejaculate was split in 4 aliquots: 1 and 2 were extended with KA and KG (1:1); 3 and 4 were centrifuged (600G), and the sperm pellet was resuspended in KA and KG. Vigor and percentage of motile sperm were determined at 24, 48 and 72 hr after storage at 5°C. Data was submitted to descriptive statistics, ANOVA and Tukey test. No differences were observed between seminal characteristics of young and adult stallions ($P>0.05$). The mean estimated values of the variables obtained were: gel-free ejaculated volume, 30 ml; sperm concentration, 101.74 x 10^6 sperm/ml; normal spermatozoa, 72.6%; total number of sperm (TEE), 2.85 x 10^9; and total viable sperm (TEV), 2.16 x 10^9. Motility of spermatozoa in both extenders was significantly lower after 24 hr of storage ($P<0.001$), and after 48 hr, sperm motility in KA was superior to KG ($P<0.05$). The cooling of plasma-free sperm in KA showed motility superior to all other treatments ($P<0.05$) at 24, 48, and 72 hr (43%, 37%, and 23%, respectively). In conclusion, there were no differences in qualitative and quantitative seminal characteristics of young and adult Crioulo stallions. The cooling of plasma-free sperm in Kenney extender with amicacin showed better motility than other treatments. Further investigations are necessary to evaluate viability and acrossomal integrity of equine plasma-free spermatozoa cooled in KA.

EFFECT OF ANTIBIOTICS ON VIABILITY AND FERTILITY OF EQUINE SEMEN COOLED TO 5^{O}C

Macedo, L.P.[1]; Papa, F.O.[1]; Gomes, G.M.[1]; Melo, C.M.[1]; Oliveira, J.V.[2]; Dell'Aqua Jr., J.A.[1]

[1]Department of Animal Reproduction- Veterinary School- UNESP- Botucatu- SP- Brazil
[2] APTA- Colina- SP- Brazil

The use of equine cooled semen has many advantages as the possibility to collect and process semen at the stud farm with subsequent transportation of the processed semen to different locations for insemination of mares. This avoids the cost and stress associated with the shipping of mares to the stud farm. Cooled semen technology has been studied for the interest in maintaining fertilizing potential of equine sample for several days. The use of antibiotics, in association or not, has been investigated aiming to verify the inhibitory action of bacterial agents in cooled semen samples. Santos et al., (2003) compared the use of different antibiotics on frozen semen and verified that amikacin sulfate (2mg/ml) inhibited bacterial growth *in vitro* and showed higher values of motility (CASA) and plasmatic membrane integrity by fluorescence staining. The aim of this study was to compare the effects of two antibiotics on the quality and fertility of equine semen cooled to 5^{o}C. Three ejaculates from five stallions from different breed were collected, aging between 5 and 17 years. Gel-free fraction was diluted (50X10^{6} spermatozoa/ml) in a skim milk-glucose extender *(Kenney et al..,Annual Conv.Am. Assoc. Equine Pract. Proceeding , p.327-336,1975)* containing gentamicin or amikacin (0.2mg/ml). The samples were analyzed daily until 96 hours for total motility, progressive motility and rapid spermatozoa (over 80μm/sec. VAP) by CASA, plasmatic membrane integrity by dual fluorescence staining describe by (*Harrison R.A., J. Reprod Fert, v.88, p. 343-352, 1990*). Data were analyzed by ANOVA and Tukey´s test. For the fertility trial 2 cycles of 12 mares were used. The mares were inseminated 36 hours after the hCG administration with 1 billion spermatozoa of semen cooled to 5^{o}C for 72 hours. On group 1 (n=12) semen was diluted in a skim milk-glucose added to amikacin (0,2mg/ml) and on group 2 (n=12) semen was diluted in a skim milk-glucose added to gentamicin (0.2mg/ml). Pregnancy was detected 15 days after ovulation by ultrasound. Total motility in amikacin samples was higher ($p<0.05$) than gentamicin on all moments evaluated. Amikacin samples showed higher values for progressive motility on 0 hour, and for rapid spermatozoa on 24 hour evaluation ($p<0.05$). From 48 to 96 hours of storage, amikacin samples had higher ($p<0.05$) total motility, progressive motility and rapid spermatozoa when compared to gentamicin samples. No statistical differences were detected on plasmatic membrane integrity evaluated by fluorescence staining between the antibiotics tested in all moments. In the fertility trial, 66.7% (8/12) of mares on group 1 became pregnant and 33.3% (4/12) on group 2 became pregnant. Despite of the higher pregnancy rate on group 1, no statistical differences were detected between the two groups. Maybe no differences were detected because of the small number of mares per group.

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EVALUATION OF PLASMATIC AND ACROSOMAL MEMBRANES INTEGRITY AND MITOCHONDRIAL MEMBRANE POTENTIAL ON DIFFERENT CONCENTRATIONS AND VOLUMES OF EQUINE CRYOPRESERVED SPERMATOZOA

Nascimento, J.[1,2]; Celeghini, E.C.C.[2]; Raphael, C.F.[2]; Andrade, A.F.C.[2]; Arruda, R.P.[2]

[1]Faculdade de Zootecnia e Engenharia de Alimentos –USP, [2]Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP, Pirassununga, SP, 13630-000, Brasil. arrudarp@usp.br

It is well known the advantages of cryopreserved semen utilization in equine reproduction, but the understanding of this biotechnology is uncertain yet, like the spermatic membranes behavior on different concentrations and straw volumes. Thus this experiment had the objective to evaluate, simultaneously, the plasmatic and acrosomal membrane integrity and mitochondrial membrane potential of equine cryopreserved spermatozoa on 100, 200 and 400x10^6sptz/mL concentrations and 0.50 and 0.25mL volumes. Eight ejaculates from four stallions with ages between 8 and 17 years old were collected with an artificial vagina. The semen was diluted in skim-milk extender (1:1), centrifuged at 500x*g* for ten minutes, the supernatant was removed and the freezing extender Botu-Crio® (Biotech-Botucatu-Ltda/ME, Brazil) was added in the pellet to a final concentrations of 100 (C100), 200 (C200) and 400x10^6sptz/mL (C400); after that, it was packed in 0.50 and 0.25mL straws. The cryopreservation of semen utilized an automatic system (TK3000®, Tekaton, Uberaba, Brazil), with cooling rate of -0.25°C/minute and freezing rate of -15°C/minute, until -80°C, and -10°C/minute, until -120°C, when the straws were plunged into liquid nitrogen (-196°C), and stored in cryogenics tank. They were thawed for 30s in a 37°C waterbath; afterward diluted in TALP at a concentration of 25x10^6sptz/mL, and a 150µL sample of this solution was added of 3µL of PI, 6µL of JC-1 and 80µL of FITC-PSA, and incubated for 8 minutes (25°C). The analysis of each sample was realized in epifluorescence microscope; it was counted 200 cells and determined the spermatozoa percentage with intact plasmatic membranes (IPM), intact acrosomal membranes (IAM), and high potential mitochondrial membrane (HPM) and intact plasmatic and acrosomal membranes and with high potential mitochondrial membrane (IPIAH). For statistical analysis were utilized variance analysis (ANOVA - $p<0.05$) and SNK test ($p<0.05$) with standard deviation, by SAS® System (Sas Institute, Cary, USA). There was no interaction between straw volume and spermatic concentration in the studied characteristics. The percentage of IPIAH cells was not different between C100 (38.4±14.98%), C200 (38.4±9.58%) and C400 (35.2±10.96%), and between 0.50 and 0.25ml volumes (37.8±12.15% and 36.9±12.06%, respectively). it was observed a statistical difference on total percentage of IPM between C100 (43.5±13.08%), C200 (40.1±9.44%) and C400 (34.9±12.43%) and between C400 and the other concentrations in HPM (46.6±11.0, 41.3±16.4% and 36.5±12.98%, respectively). for IAM no differences were observed between the different studied concentrations (81.03±7.09%, 83.1±17.0% and 78.0±12.97%). between 0.50 and 0.25 mL straws, there weren't statistical difference to ipm (40.65±11.1% and 38.55±13.08%), IAM (82.4±7.55% and 79.3±16.3%) and HPM (41.2±13.09% and 38.06±14.15). We concluded that C100, C200 and C400 maintain equally the IPIAH and IAM cells integrity; C100 and C200 preserve higher percentage of IPM and C100, C200 and C400 maintain, orderly, larger percentage of IPM.

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EFFECTS OF SPERMATIC CONCENTATION AND STRAW VOLUME IN MOTION CARACTERISTICS OF EQUINE CRYOPRESERVED SPERMATOZOA EVALUATED BY COMPUTER SYSTEM – CASA

Nascimento, J.[1,2]; Celeghini, E.C.C.[2]; Andrade, A.F.C.[2]; Raphael, C.F.[2]; Arruda, R.P.[2]

[1]Faculdade de Zootecnia e Engenharia de Alimentos – USP; [2]Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP; Pirassununga, SP, 13630-000, Brasil. arrudarp@usp.br

The use of equine cryopreserved semen has increased in importance and frequency; however there was not agreement of the ideal spermatic dose and concentration to optimize fertility results. Some cryopreservation protocols have been developed to reach better results, like the combination of different stored volumes and spermatic concentrations. This experiment proposed to compare two straw volumes (0.50 and 0.25mL) and three spermatic concentrations (100, 200 and 400x10^6sptz/mL), using the spermatic motility computerized analyzer (CASA). Eight ejaculates from four stallions with ages between 8 and 17 years old were collected in artificial vagina. The semen was diluted in skim-milk extender (1:1), centrifuged at 500x*g* for ten minutes, the supernatant was removed and the freezing extender Botu-Crio® (Biotech-Botucatu-Ltda/ME, Brazil) was added in the pellet to a final concentration of 100 (C100), 200 (C200) and 400x10^6sptz/mL (C400); after that, it was packed in 0.50 and 0.25mL straws. The cryopreservation of semen utilized an automatic system (TK3000®, TK Tecnologia em Congelação Ltda, Uberaba, Brazil), with cooling rate of -0.25°C/minute and freezing rate of -15°C/minute, until -80°C, and -10°C/minute, until -120°C, when the straws were plunged into liquid nitrogen (-196°C), and stored in cryogenics tank. Two straws from each treatment were thawed for 30s in a 37°C waterbath; afterward they were diluted in TALP to a concentration of 25x10^6sptz/mL for CASA evaluation. The analyzed characteristics were: total motility (TM, %), progressive motility (PROM, %), progressive velocity (VSL, µm/s), track speed (VCL, µm/s), beat cross frequency (BCF, Hz) and lateral amplitude of head (ALH, µm). For statistical analysis were utilized analysis of variance (ANOVA - $p<0.05$) and SNK test ($p<0.05$) with standard deviation, by SAS® system (SAS Institute, Cary, USA). There was not interaction between straw volume and spermatic concentration in the studied characteristics. The TM and PROM showed statistical differences among C100, C200 and C400 (47.85±10.76% and 16.63±6.45%; 38.44±12.20% and 10.92±6.14%; 31.40±12.70% and 7.45±5.65%, respectively). The 0.5 and 0.25mL straws resulted in a similar TM and PROM (39.92±14.10% and 11.30±7.55%; 38.41±13.20% and 11.97±6.71%, respectively). ALH and BCF presented statistical differences among C100, C200 and C400 (5.13±0.40µm and 38.55±1.52µm; 4.56±0.54µm and 35.4±1.98µm; 4.06±0.56µm and 33.24±2.02µm, respectively); however there were no statistical differences between 0.5 and 0.25mL straws (4.5±0.64µm and 35.8±2.80µm; 4.6±0.69µm and 35.5±2.90µm, respectively). VSL and VCL had statistical differences among C100, C200 and C400 (77.77±6.10µm/s and 154.77±12.20µm/s; 65.50±8.40µm/s and 129.10±16.40µm/s; 56.68±8.90µm/s and 110.37±17.60µm/s, respectively) and equally in 0.5 and 0.25mL straws to VCL (65.90±11.98µm/s and 67.10±22.90µm/s, respectively). Thus, the better freezing method, in this experiment, was C100, followed by C200, and C400, in all studied variables, as well in 0.5 as 0.25mL straws, with exception to VCL that presented better result in 0.25mL.

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FERTILITY RATES OF EQUINE SEMEN COOLED FOR 24H BEFORE FREEZING

Melo, C.M.[1]; Zahn, F.S.[1]; Martin, I.[1]; Trinque, C.L.A.[2]; Alberti, K.[1]; Orlandi, C.[1]; Siqueira-Filho, E.R.[1]; Dell'Aqua Jr., J.A.[1]; Alvarenga, M.A.[1]; Papa, F.O.[1]

[1]Department of Animal Reproduction and Veterinary Radiology- UNESP Botucatu SP Brazil; [2]Equestre CenterAgromen Orlândia – SP; papa@fmvz.unesp.br

Although equine semen cryopreservation is a relatively simple technique and can be done at any field condition; it is certainly necessary sophisticated equipment and an experienced technician. Despite of sufficient results, this technique has been presenting many inherent problems as the risk of stallion management, use of adequate extenders, equipment cost and transportation to the farm and finally a standardized protocol. Based on these aspects, the development of a new protocol using cooled semen for freezing would minimize those problems. Thus, semen would be collected at stallion farm, handling as cooling semen and then transported to an equipped laboratory for concluding freezing process. Thus, the aim of this study was to verify the characteristics of equine frozen semen previously cooled for 24h before freezing. One ejaculate from each of 13 stallions (4-18 years) was collected and first evaluated by CASA. Semen sample was divided into two parts; one part was frozen following a regular protocol (Papa *et al*, **Rev Bras Reprod Anim**, v.26, n.3, p.184-187, 2002*)* and the other part was diluted with Botu-Semen® to 50 x10^6 motile spermatozoa/mL and then stored at Equitainer® for 24 hours. Afterwards, cooled semen was centrifuged at 600x*g*/10 minutes. Supernatant was removed and Botu-Crio® freezing extender added at 20ºC. Semen was packed in 0,5 mL straws, frozen 6 cm above liquid nitrogen for 20 minutes and finally plunged into liquid. After thawing, semen was submitted at CASA evaluation and for plasma membrane integrity by fluorescent probes (Harrison R.A., **J. Reprod Fert**, *v*.88, p. 343-352, 1990). Fertility trial was carried out using frozen semen from two stallions. Twenty-two mares were inseminated 43 times. Mare's estrus was followed by rectal palpation and ovary ultrasonography and the ovulation was induced using 10 mg/EPE (equine pituitary extract) intravenously. Inseminations were performed twice, one before and one after ovulation (within 6 h of ovulation) with 350 x 10^6 spermatozoa/mL toward the tip of the horn by a flexible pipette (MiniTüb ®). Data about semen and fertility rate were studied by using ANOVA (GLM procedure of SAS, SAS, Institute, Inc., Cary, NC, USA). Significance was set at $P < 0.05$.There were no statistical differences on semen parameters: total motility, progressive motility, plasma membrane integrity and pregnancy rates when comparing the two methods for freezing semen. Pregnancy rates for conventional and cooled/frozen semen were 72.7% and 82.3% (stallion A) and 40.0% and 50.0 (stallion B) respectively. Thus, this new approach for freezing semen previously cooled for 24h could be the manner of reducing many problems related to risk of transporting expensive equipment or even the stallion for one location to another. These results indicate that cooling equine semen for 24h before cryopreservation maintained sperm viability and fertility, arising a new biotechnology for equine semen.

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EFFECT OF DIFFERENT CONCENTRATIONS OF CRYOPROTECTORS ON EQUINE SEMEN FREEZING

Melo, N. S.S.[1]; Pimentel, C.M.[1]; Silveira, L.L.[1,2]; Mariante, A.S.[2]

[1] Universidade de Brasília-UnB/Faculdade de Agronomia e Medicina Veterinária-FAV, 70910-900 Brasília-DF, Brasil; [2] Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, 70770-900 Brasília-DF, Brasil. soternatalie@yahoo.com.br

Spermatozoa cryopreservation has used since the 1950's, however not many experiments with horses have being carried out. The objective of this study was to evaluate the effect of the cryoprotectors Glycerol (G) and Dimetylformamide (DF), in different concentrations, on equine semen freezing, through tests of membrane integrity and motility. Five Mangalarga Marchador stallions were utilized, weighing between 400 to 550 kg, and ages varying from 4 to 23 years. The following concentrations were used: G 4% (T1), G 3% + DF 1% (T2), G 2% + DF 2% (T3), G 1% + DF 3% (T4) e DF 4% (T5). During the cooling and freezing processes, the freezing machine model TK 3000® was utilized. Samples were thawn in a water bath at 40ºC for 30 seconds, and analyzed for optical motility and vigor, with a hyposmotic test as well as test utilizing fluorescent probes were carried out. The results showed that there were significant differences ($p<0.001$) between stallions and treatments in relation to hyposmotic test (16.77%), fluorescence (49.60%) and motility (34.06%), as well as an interaction between stallion*treatment affecting membrane integrity, measured by both hyposmotic (12.56%) and fluorescence tests (2.85%). The dimetylformamide was shown to be a good option as a cryoprotector for freezing equine semen, since the semen presented better quality, when there was an increase in the percentage of this cryoprotector in the freezing solution and, consequently, a reduction in the Glycerol percentage. This increase in semen quality may be due to the known toxicity of Glycerol.

THE INFLUENCE OF DIFFERENT CRYOPROTECTANTS AND ITS ASSOCIATIONS ON EQUINE FROZEN SEMEN

Dores, C.B.[1]; Papa, F.O.[1]; Blanes, M.[1]; Melo, C.M.[1]; Dell'Aqua Jr., J.A.[1]; Alberti, K.[1]; Crocci, A.J.[2]

[1] Department of Animal Reproduction and Veterinary Radiology FMVZ- Unesp, Botucatu
[2]Department of Biostatistic - IB UNESP, Botucatu, papa@fmvz.unesp.br

Although there have been constant researches on equine frozen semen, fertility rates remain about 50% for many years. Glycerol has been used since 1949, however it has toxic effects on spermatozoa which stimulated recent studies using amides as alternative cryoprotectants. The aim of the present study was compare two extenders for equine semen cryopreservation: Botu-Crio™ (Biotech-Botucatu-Ltda/ME, Brasil) and FR4 (Palmer E., CONGRESS ON ANIMAL REPRODUCTION, AI, 10, Champaign, IL. *Proceedings* Champaign. p. 769, 1984)added with association between different amides and glycerol. Three ejaculates from 5 stallions of different breeds from Animal Reproduction Area (Department of Animal Reproduction and Veterinary Radiology – FMVZ/UNESP – Botucatu-SP). Semen was evaluated before and after cryopreservation. Sperm motility parameters were evaluated using computer-assisted sperm analysis system and membrane integrity by fluorescent probes (*Harrison R.A., J. Reprod Fert, v.88, p. 343-352, 1990*). Semen was diluted with Botu-Crio™ and centrifuged in multiples tubes at 600xg for 10 minutes. Supernatant was removed and Botu-Crio™ (Biotech-Botucatu-Ltda/ME, Brasil) freezing extender added to the remaining pellets with Botu-Crio™ (Biotech-Botucatu-Ltda/ME, Brasil) containing: methyl formamide 4% (M1); methyl formamide 3% + glycerol 1% (M2); glycerol 3% + methyl formamide 1% (M3); dimethyl formamide 2%+ methyl formamide 2% (M4); and FR4 (INRA 82, Palmer, 1984) 2.5% methyl formamide + 2.5% dimethyl formamide (M5) adjusted to $200x10^6$ sperm/mL, packed in 0.5mL straws. The straws were cooled at 5ºC during 20 minutes then frozen 6 cm above liquid nitrogen for 20 minutes and finally plunged into liquid. Straws were thawed in a 46ºC water bath for 20s prior to analysis. Data about semen were studied using ANOVA and Tukey. Significance was set at $P < 0.05$. Results for total and progressive motility, and membrane integrity after thawing for the different treatments were respectively: M1 (47.2^{ab} / 21.1^{a} / 48.1^{ab}); M2 (48.7^{a} / 21.4^{a} / 53.2^{a}); M3 (40.9^{ab} / 18.9^{a} / 48.5^{ab}); M4 (38.8^{b} / 18.1^{a} / 45.8^{b}); M5 (43.2^{ab} / 18.4^{a} / 42.8^{b}). Based on these results, Botu-Crio™ (Biotech-Botucatu-Ltda/ME, Brasil) and cryoprotectants associations used at treatments M1, M2 and M3 had greater results after thawing. The association between methyl formamide 3% + glycerol 1%was the most efficient on maintaining sperm viability after cryopreservation. The present study concluded that the association of different cryoprotectants and extenders protected spermatozoa from cryodamage, which can provide an alternative for those bad freezer stallions.

Acknowledgments:
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EFFECT OF SPERM CONCENTRATION AND STABILIZATION TIME ON EQUINE FROZEN SEMEN USING BOTU-CRIO® EXTENDER

Blanes, M.S.[1]; Papa, F.O.[1]; Dores, C.B.[1]; Melo, C.M.[1]; Crocci, A.J.[2]

[1]Department of Animal Reproduction and Veterinary Radiology FMVZ- Unesp, Botucatu
[2]Department of Biostatistic - IB UNESP, Botucatu; papa@fmvz.unesp.br

The reasonable results of frozen semen are the most important limitation of its use. One important point that must be considered is the spermatozoa concentration inside the straws. The aim of the present study was to evaluate the influence of spermatozoa concentration packed in 0.5mL straws and compare two times of stabilization at 5ºC (20 and 60 minutes), before freezing procedures, using Botu-Crio™ extender. Five stallions from different, breed belonging to Animal Reproduction Area - Department of Animal Reproduction and Veterinary Radiology, FMVZ/UNESP Botucatu – Brazil were used. Three ejaculates from each stallion were collected and submitted to CASA and membrane integrity was evaluated using fluorescent probes (*Harrison R.A., J. Reprod Fert, v.88, p. 343-352, 1990*). After the determination of total number of spermatozoa in the ejaculate, the semen was diluted using Botu-Semen® and centrifugated at 600xg for 10 minutes. Supernatant was removed and Botu-Crio® freezing extender added, and straws were packed with different concentrations of viable sperms: $200x10^6$ (C1), $150x10^6$(C2), $100x10^6$ (C3) and $50x10^6$ (C4). Part of straws were submitted to stabilization for 20 minutes and part submitted to 60 minutes, frozen 6 cm above liquid nitrogen for 20 minutes and finally plunged into liquid. After thawing at 46ºC/20", semen was submitted again at CASA evaluation and for plasma membrane integrity by fluorescent probes (*Harrison R.A., J. Reprod Fert*, v.88, p. 343-352, 1990). Data about semen were studied by using ANOVA. Significance was set at $P < 0.05$. There were no statistical differences on semen parameters: total motility, progressive motility, plasma membrane integrity. Sperm parameters for C1, C2, C3 and C4 total motility (57.07; 61.20; 59.20 e 57.33), progressive motility (22.0; 23.3; 22.3 e 22.8) and membrane integrity: (54.1; 56.1; 57.5 e 57.6) respectively. Differences were observed only on total motility when the two periods of stabilization at 5ºC were compared. Sperm parameters for 20 and 60 minutes were respectively: total motility (52.80^a e 43.55^b), progressive motility (22.50^a e 17.10^a) and membrane integrity (50.60^a e 51.5^a). The present study concluded that there was no difference on sperm viability *in vitro* when different numbers of sperm straws were used. However, 20 minutes of stabilization at 5ºC before freezing was better when compared with 60 minutes with the utilization of Botu-Crio® extender.

Acknowledgments:
Financial support FAPESP
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EFFICIENCY OF EQUINE CRYOPRESERVED SEMEN APPLICATION IN ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER PROGRAM.

Granito, A.L.[1,2]; Nascimento, J.[2]; Arruda, R.P.[2]

[1]Haras Vila dos Pinheiros Ltda, Indaiatuba, SP, Brazil; [2]Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP; Pirassununga, SP, 13630-000, Brasil. vetgranito@hotmail.com

The use of equine cryopreservation semen in artificial insemination and embryo transfer programs has brought many advantages to equine reproduction, as the better utilization of animals and the accelerated improvement of breed. The objectives of this work were: 1) to compare the results between recovery embryo rate and pregnancy rate in mares that were inseminated artificially with equine cryopreserved semen and 2) to compare the results of pregnancy rate and embryo loss rate between mares received embryo or were inseminated artificially with equine cryopreserved semen. The experiment was carried out during breed season 2004/05 of Stud Vila dos Pinheiros, Indaiatuba, SP, using cryopreserved semen of one stallion and mares Arabian Horse breed. Twenty and two mares were used; eight were directed to embryo recovery and transfer (G1) and fourteen were inseminated to maintained pregnancy (G2). The mares of both groups were teased each 48 hours and the mares in estrous were examined by ultrasound regard to uterus and ovarian follicles development. Mares with high uterus ecogenicity (endometrial edema) and ovarian follicles = 35mm received 2,500IU of hCG (Vetecor®, Calier, Barcelona, Spanish), by intravenous, and then, examined each 12 hours in the first 24 hours and each 6 hours in last 24 hours. Immediately before detection of ovulation, it was released A.I. in junction utero-tubaric ipsilateral at ovulation with cryopreserved semen, stored into 2mL macrotube and thawed for one minute in 40°C waterbath (=300x106spermatozoa with motility). In G1, the embryo recovery was performed 08 days after A.I., then embryos were transferred into recipient ovulated two days before donor mares. The pregnancy diagnosis of mares that received embryos from G1 and the pregnancy diagnosis of mares inseminated artificially (G2) were realized at 14 (D14) and 60 (D60) days after ovulation, by ultrasound. The data of first estrous cycle were analyzed by Chi Square test (?2), by SAS® system (SAS Institute, Cary, USA). There was no statistic difference (P>0.05) between recovery embryo rate (G1) and pregnancy rate at D14 (G2), whose results were 62.5% (5/8) and 71.4% (10/14), respectively. The results of pregnancy rate at D14 and D60 of mares that received embryo and mares that were inseminated were 80.0% (4/5) and 40.0% (2/5); 71.4% (10/14) and 50.0% (7/14), resulting in loss embryo rate of 50.0% and 30.0%, respectively. There was no difference belong the treatments (P>0.05). The results indicate the equine cryopreserved semen can be utilized in artificial insemination and transfer embryo program, nevertheless more atention must be directed to loss embryo factors.

EFFECT OF BODY WEIGHT OVER SEXUAL DEVELOPMENT PARAMETERS IN SANTA INEZ LAMB RAMS

Siqueira Filho, E.R.[1]; Alberti, K.[1]; Bicudo, S.D.[1]

[1]Departament of Animal Reproduction and Veterinary Radiology, FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. ersiqueirafilho@hotmail.com

The significant increase in sheep raising in the state of São Paulo, the technification and the search for higher productivity require an increase in fertility and in prolificity of herds. One of the highest points for this increase in raisings is the selection of more precocious and prolific animals, by shortening the space between progenitures and by promoting genetic improvement of herd. The testicle size estimated by the scrotal circumference (SC) is an excellent indicator for reproductive potentiality of rams (Moraes e Oliveira, Rev. Bras. Reprod. Anim. v.16, p.55-62, 1992) and is clearly associated to body weight (BW) (Salgueiro e Nunes, Rev. Bras. Reprod. Anim. v.23, p.231-232, 1999). The excessive deposition of scrotal fat (SF) interferes negatively in thermoregulatory mechanisms, which are essential for spermatogenesis, relating to a highly energetic diet (Fourie et al., Small Ruminant Research v.54, p.53-59, 2004) and consequent increase in BW. The testicular measurements weight (TW) and volume (TV) are associated to spermatic production in rams (Souza e Costa, *Anais* Simpósio em Ciências Agrárias, Teresina-PI, p.80-86. 1992). This study aimed to correlate body weight to sexual development indicators SC, TV and TW and scrotal fat deposition, thus helping sheep breeders to precociously select animals for reproduction. 13 male Santa Inez lamb rams, born in June 2004, confined for 40 days from August 18, 2004, were evaluated. Live weights of animals were measured by using an electronic scale (Kg), SC was measured by using a metallic meter tape (cm), with the animal standings. Scrota and testicles were collected after slaughtering, in a refrigerating chamber. Weight of testicles was measured in analytical scale, after removal of epididymis, and, next, volume was calculated by the equivalent liquid dislocation. Scrota were stored in freezer and defrosted for fat removal, which was weighted in analytical scale. Correlation estimations were determined by the Pearson method ($P<0.01$). BW, SC, TV, TW and SF averages obtained were: 36.1 kg, 25.2 cm, 154.2 mL, 140.6g and 27.1g, respectively. Correlations between BW and SC ($r=0.82$), TV ($r=0.68$) and TW ($r=0.66$) found, as well as SC and TV ($r=0.72$) and TW ($r=0.73$) and TV and TW ($r=0.99$) presented themselves high, showing that such parameters may be used as sexual development indicators since youth. Correlations of variables SF and BW ($r=0.23$) and SC ($r=0.09$) presented themselves low, showing that the deposition of scrotal fat at the age evaluated is not significant in the development of sexual parts of lamb rams.

PROTEINS AND CHOLESTEROL OF RAM SEMINAL PLASMA WITH DIFFERENT LEVELS OF FREEZABILITY

Câmara, D.R.[1,2]; Simplício, K.M.M.G.[1]; Porto, A.L.F.[1,3]; Padilha, R.T.[2]; Cavalcanti, M.T.H.[3]; Moreira, K.A.[3]; Guerra, M.M.P.[1]

[1]PPGCV/UFRPE. Rua D. Manoel de Medeiros s/n. Dois Irmãos. Recife-PE. Brasil. [2]FMV/CESMAC. [3]LIKA/UFPE. diogocamara1979@hotmail.com

The discovery of seminal plasma proteins able to modulate cholesterol content of spermatic membrane, beyond others that are adsorbed to spermatic cells exerting a protection effect, can have a relation with semen freezability. This work aimed to identify the profile of protein and cholesterol from ram seminal plasma, Santa Inês breed, with different levels of freezability, after semen cryopreservation. The samples from nine rams in a flock located in the northeast of Brazil (9º19'06" South; 35º33'06" East) kept in similar handled and feeding conditions were collected by artificial vagina and diluted with Fiser. After post-thaw evaluation, the animals were divided into three groups, denominated high (H, 80% ejaculates approved), intermediate (I, 60% ejaculates approved) and low (L, 60% ejaculates approved) freezability. The samples were considered approved post-thawed with 30% progressive motility at minimal. Thereby, twelve samples of semen from each ram were collected by artificial vagina and centrifuged at 950 x g/30 min, at room temperature. The supernatant was removed and sent under refrigeration to laboratory and stored at –20ºC, until total protein (TP), cholesterol (TC) and high density lipoproteins (HDL) analysis were performed using commercial kits (Labtest). For the SDS-PAGE, using 10% acrylamide gel the seminal plasma samples were again centrifuged (6,000 x g/60 min at 4ºC) and refrozen at -20 ºC until analysis. Immediately before electrophoresis, samples were thawed at room temperature, extended on buffered solution and denatured. The molecular weight standards used were bovine serum albumin, ovoalbumin, carbonic anhydrase, trypsinogen, trypsin inhibitor and α-lactoalbumin. After electrophoresis, the gels were stained overnight in 0.25% Comassie Brilliant Blue, 45% methanol and 10% acetic acid in distilled water. Gels were destained in 45% methanol and 10% acetic acid in distilled water. The molecular weight of protein bands were determined by a logarithmic curve model, based on migrations of bands from standards of molecular weight. Data from seminal plasma biochemical and fresh/frozen semen profile were analyzed using ANOVA. The relations between semen freezability and electrophoresis were evaluated by descriptive analysis. There weren't statistical differences for TP, however rams from H and I groups showed lower concentration of TC and HDL in the seminal plasma than rams from the L group ($P < 0.05$). The eletrophoretic profile identified 18 bands in 2D-SDS, with molecular weight from 16.4 to 78.8 kDa. One of the bands with 24.3 kDa molecular weight was absent in 83.33% of samples from low freezability group, however it was detected in 66.67% of samples from high and intermediate freezability groups. In conclusion, the 24.3 kDa protein detected in seminal plasma of Santa Inês rams can exert a membrane protect effect, decreasing the cholesterol efflux in spermatic cells during cryopreservation process.

INTRAUTERINE ARTIFICIAL INSEMINATION IN SHEEP THROUGH THE CERVICAL VIA

Silva, J.C.B.[1]; Oliveira, R.[1]; Schneider, C.[1]; Traldi, A.S.[2]

[1]Embryolife, Ouro Fino- MG, 37570 000, Brasil. [2] Departamento de Reprodução Animal – FMVZ/ USP, Pirassununga, SP, 13630 000, Brasil. juliovet@overnet.com.br

The pronounced cervical folds make difficult the artificial insemination in sheep through de cervical via. In addition, fertility after frozen-thawed semen of this species shows low results, therefore, this procedure is not recommended for cervical insemination. The development of a technique of intrauterine artificial insemination by pulling and exteriorizing the uterine neck, allowing passing the straw gun across cervical rings, could make possible to use frozen semen. In order to validate such a technique, 81 Santa Inês ewe, were inseminated with semen from different Dorper rams. These females were synchronized with 60 mG medroxiprogesterone acetate vaginal pessary, which was withdrawn 9 days later. A 400 IU of eCG was given on the 7th day of treatment and insemination took place 55 hours after sponge withdrawal using fresh semen extend in skim UHT cow milk, packed into 0.25 mL French straws. Ewes were restrained standing. A human duck bill type speculum coupled to a light source aided to identify the cervix, wich was clamped and pulled out by a Posy tweezers, allowing its exteriorization and introduction of the metallic straw gun. This applicator is 7 mm long and has a blunt tip of 2 mm diameter, which facilitates to pass through cervical rings and allows performing the insemination either in the body or in the neck of the uterus, preventing injuries. Pregnancy was diagnosed by transretal ultrasonography and showed a total of 48 pregnant ewes (59.25% - 48/81) of which 79.2% (34/48) from intrauterine insemination (IU), 12.5% (6/48) from deep deposition into the cervix (DC) and 8.3% in the middle of the cervix (MC). Efficacy of the method was 70.4% of IU, 14.8% of DC, 13.6% of MC and only 1.2 % for superficial depositions. Results showed that cervical traction associated to the peculiarities of the straw gun facilitate intrauterine deposition of semen, which may represent a promising strategy to the use of frozen semen in Santa Inês breed.

USE OF SEMINAL PLASMA TO THAW RAM SEMEN FOR TRANS-CERVICAL ARTIFICIAL INSEMINATION (AI) IN EWE WITH SYNCHRONIZED ESTRUS

Silva, T.A.S.N.[1]; Neves, J.P.[1]; Bragança, J.M.[2]; Gonçalves, P.B.D.[2]; Rumpf, R.[3].

[1] Faculdade de Agronomia e Medicina Veterinária da UNB – Brasília – DF thiagoasns@unb.br [2] Biotecnologia e Reprodução Animal – BioRep – UFSM [3] Embrapa Recursos Genéticos Biotecnologia – Brasília – DF

The aim of the present experiment was to evaluate the effect of the seminal plasma for thawing ram semen in pellets on trans-cervical artificial insemination (AI) in sheep with synchronized estrus. Ewes (n=161), Polwarth bred, gynecological health, with body condition above 3 (1-5), raised in São Gabriel - RS, Brazil, were inseminated on February, 2005. The ewes had their estrus synchronized with a vaginal device containing 50mg of medroxyprogesterone acetate (MPA) by 12 days. The animals were injected IM with 250UI of eCG (Novormon) when the MPA was removed. The semen was obtained from five Polwarth ram and frozen in TRIS diluent with citric acid, glucose and egg yolk in a minimal concentration (1:2) of 200 x 10^6 cells per dose in pellets 130 µl. A sample of each batch was submitted to thermo-resistance test (TRT; 37ºC/6 hours) with and without ram seminal plasma and the acrosome integrity was evaluated using Tryplan blue/Giemsa stain. Semen samples were used when they were with a mobility of at least 25% in TRT and 20% of live cells and acrosome integrity after 6 h with and without seminal plasma. The seminal plasma obtained from two rams, after collection was centrifuged and preserved -20ºC. The seminal plasma was defrosted close to the pellet in bathe Maria to 37ºC in the proportion of (1:1). The AI was in fixed time, at 50 hours after removing the vaginal device, using vaginoscpy with light source and semen applicator for 0.25ml straws. The penetration degree of the semen applicator in the ewe cervical channel was classified as following: degree 1: superficial; degree 2: intermediate; degree 3: deep. The ewes in control group were inseminated with fresh semen diluted with citric acid/glucose. The other 100 ewes were divided in two groups: inseminated with frozen semen and thawed with or without seminal plasma. The diagnosis of pregnancy was performed 45 days after AI with ultrasound. The pregnancy rate was 60.0% (50/30), 74.0% (50/37) and 67.2% (61/41), respectively for frozen semen and thawed without seminal plasma, with seminal plasma and fresh semen. In according to cervical penetration, the percentage of pregnancy was 52.3% (Degree 1), 66.6% (Degree 2) and 83.3% (Degree 3). The pregnancy rate did not differ among treatments in degrees 1 and 2. However, the pregnancy in ewes inseminated with fresh semen (95.6%) and with frozen semen/thawed with plasma (94.1%) were higher than with frozen semen/thawed without plasma (60.0%; $P<0.05$) in degree 3. In conclusion, frozen semen can be used by via cervical with similar pregnancy rate of fresh semen in degree 1 and 2. However, in degree 3, pregnancy rate similar to fresh semen is only obtained when frozen semen is thawed with seminal plasma.

ARTIFICIAL INSEMINATION OF GOAT USING SEMEN EXTENDER AND CRYOPRESERVED WITH TES.

Silva, A.O.A.[1]; Sousa, J.S.[1]; Reis, A.N.[1]; Oliveira, C.M.C.[1]; Silva, L.K.X.[1]; Vale, W.G.[2]; Ribeiro, H.F.L.[2]

[1]Central de Biotecnologia de Reprodução Animal - UFPa, Castanhal-Pa; [2]Universidade Federal Rural Amazônia - UFRA, Belém-Pa. otavio@ufpa.br

The objective of the experiment was to evaluate the efficiency of the TES as extender for the semen of goat. It was performed in Castanhal city, state of Pará. Two males of the Alpine, 7 crossbred females and 4 females Alpine pure were used, with age varying between 2 and 3 years. The oestrus synchronization was performed through of the application in day 0 of impregnated vaginal sponges with 60 mg of Acetate of Melengestrol, that remained in the animal per 13 days. In 13th day was applied 300 UI of eCG. The oestrus observation was performed in 14th and 15th day of the implantation of the vaginal sponges. The semen was collected through artificial vagina, and was evaluated sperm motility and vigor, then a sample of 10 and 20 µL of semen was used for evaluation of the pathology and the concentration spermatic. The semen then was diluted in TES (hydroxymethy ethyl amino ethanesulfonic acid), contents as cryoprotector the Glycerol (7%), to a concentration of 50 x 10^6 sptz/dose. The diluted semen was loaded in 0.5 ml straws, and frozen in liquid nitrogen. The motility and vigor of the semen were observed, in the fresh semen, post-diluition and post-thawed (90%/4, 90%/4 and 60%/4, respectively). The semen were deposited directly into the cervix, 12 and 24 h after the sponge withdrawal. The diagnosis of the gestation was performed 45 days after insemination, through ultrasonography. The fertility of the cryopreserved semen was determined through the pregnant percentage that it was of 90.9% (10/11). The parturition percentage was of 72.7% (8/11). It was concluded that the diluent TES can be used with efficiency for the cryopreservation of the semen of goat.

CRYOPRESERVATION OF RAM SEMEN IN TREHALOSE SOLUTION

Valleriote, P.S.[1]; Dias, A.J.B.[1]; Paes de Carvalho, F.[1]; Paes Sobrinho, C.[1]

[1]Laboratory of Improvement Genetic Animal /LMGA/ CCTA/ UENF,
Campos dos Goytacazes-RJ, 28013-600, Brazil. valleriote@yahoo.com.br

The plasma membrane of the ram spermatozoa has a particular composition what makes difficult its cryopreservation with efficiency. Therefore, the frozen semen use in programs of artificial insemination, with cervical deposition, needs the development of extenders that improve the pregnancy rates. Trehalose has a cryoprotective action, because its hipertonic effect, what it diminishes the intracellular water and therefore the amount of injuries for the ice crystal formation during the freezing/thawing. Moreover trehalose confers protection to the spermatozoa through the specific interactions with membrane phospholipids. For not crossing the plasma membrane does not present harmful effect to the spermatozoa in concentrations of up to 100 mOsm. Thus, the objective of this work was to evaluate the effect of the addition of trehalose in ram semen extender, in order to get adequate cellular integrity after thawing. Ejaculates of the two rams Santa Inês had been collected with aid of a artificial vagina (IMV Technologies) and diluted in TRIS-egg yolk (Tris, citric acid, glucose, egg yolk, glycerol), added or not of trehalose 0.01M. The cooling was carried through in a programmable freezer (Haake C-760) 0.05°C/min., during 2h, until reaching 5°C. In this temperature the addition of glycerol in three stages was carried through, until getting a final concentration of 7%. After two hours the straws contend $50x10^6$ spermatozoa/mL had been placed in the nitrogen vapor a distance of 6 cm of the surface, for a period of 4 minutes and later immersed in liquid nitrogen. The evaluation of the tolerance to the freezing was based on the motility, vigor and in the hiposmotic test. The average values of seven repetitions demonstrated great reduction of the motility after thawing, of similar form for samples frozen in the absence or presence of trehalose (42.0% ± 10,7 and 48% ± 7,0, respectively). However it did not have important difference in the vigor, in all the observed samples, remaining with score 4,0 (scales of 0 the 5). In the same way the addition of trehalose did not modify the results to the hiposmotic test, of the frozen spermatozoa in the absence (27% ± 9.6) or in the presence (29.5% ± 5,0) of trehalose. The quality of the semen samples used in this experiment could have contributed to the lower results obtained, since the average of motility of the fresh semen samples was 76.0%. In the conditions of this work the addition of 0.01M of trehalose did not promote significantly improve in the motility rates and preservation of the integrity of the plasma membrane of the frozen spermatozoa.

EFFECT OF EQUEX-STM ADDICTION IN EGG YOLK-TRIS EXTENDER ON THE MOTILITY OF FROZEN-THAWED RAM SPERMATOZOA

Maia, M.S.[1]; Azevedo, H.C.[2]; Bicudo, S.D.[3]; Sousa, D.B.[3]; Rodello, L.[3]

[1]Embrapa Semi Árido; [2]Embrapa Tabuleiros Costeiros; [3] Animal Reproduction and Veterinary Radiology Department – FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. sony@fmvz.unesp.br

This study was designed to evaluate the effect of addition the detergent Equex-STM to the extender on the post thaw motility of ram spermatozoa. Twenty ejaculates were obtained from 10 Santa Inês rams. The freezing protocol involved diluting the semen in two steps, at 32°C using egg yolk-Tris extender (3.028g de Tris- hydroxymethyl- amino methane, 1.675g of citric acid monohydrate, 0.2g de glucose, 20mL egg yolk in 100mL double distilled water and 7% of glycerol) containing 0; 0.5 or 1.0% of Equex-STM. The semen was packaged in 0.25mL straws, frozen in an automatic freezing machine (Tetakon®-TK 3000) and the sperm motility was evaluated using a computer-assisted sperm analysis (CASA). Equex-STM supplementation increased significantly ($p<0.05$) both total and progressive spermatozoa motility. In the extender Tris 0% the total motility was 34% and the progressive motility 24%, while in the extender Tris 0.5% they were 65% and 39%, and in the Tris 1% they were 70% and 39%. The Equex-STM showed a beneficial effect on the sperm motility when added to the egg yolk-Tris extender on the concentration of 0.5 or 1%, it is suggested that this extender may be used for the sheep semen frozen.

INFLUENCE OF THE TEMPERATURE IN THE TESTICULAR DEGENERATIVE PROCESS OF BULLS IN REGIMEN OF SEMEN COLLECTION

Silva, L.K.X.[1]; Silva, A.O.A.[1]; Reis, A.N.[1]; Sousa, J.S.[1]; Ribeiro, H.F.L.[2]

[1]Central de Biotecnologia de Reprodução Animal – UFPA, Castanhal-Pa; [2]Universidade Federal Rural da Amazônia – UFRA, Belém-Pa. silva_lilian@yahoo.com.br

The objective of this work was to evaluate the effect of the environment temperature in the thermoregulatory mechanism of testes and in the production and quality spermatic of *Bos indicus* and *Bubalus bubalis*. Were used six bulls, being three *Bos indicus* (Group A) and three *Bubalus bubalis* (Group B). In the days of semen collection, the temperature was measured, in three different schedules (07h:00min, 13h:00min and 17h:00min), of the head and the tail of epididymis, of the posterior face of the testes, of the vascular cone of both the testes, and also the temperature of the region near to the spike and to femur. The temperature was verified through a digital thermometer the laser (Model RAYNGER ST6TM). The semen was collected through artificial vagina, being ten samples of each animal. After the collection, the semen was evaluated in relation to the volume, color/density, wave motion, motility, vigor, concentration and sperm morphology. The results showed that in the group A and group B, it did not have difference statistics in relation to the temperature verified in the different schedules, of the head of epididymis, the tail of epididymis, of the central part of the testicule, the vascular cone and the body temperature. As statistical method it was used the analysis of variance with level of significance of 5%, and the test of Tukey. Through the analysis of the semen it was observed that the spermatic morphology, the motility, the vigor and the wave motion had also not suffered influence from the surrounding average temperature, whose average was around 26.8ºC. The average of the spermatic morphology, motility, vigor and wave motion in the group A was of 62.3%, 77.7% , 3.3 and 3.3, and in the group B was of 72.1%, 68.8%, 3.1 and 3.1. In conclusion, the temperature testicular, the quality of the semen and the spermatic production had not suffered influence from the surrounding average temperature enter the groups A and the B, and inside of the groups.

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TESTICULAR BIOMETRY AND HISTOPATHOLOGY OF NELORE BULLS

Goiozo, P.F.I.[1]; Di Santis, G.W.[2]; De Moura, V.M.B.D.[3]; Bandarra, E.P.[2,3]

[1] Embrapa Beef Cattle. Campo Grande- MS, 79002-970, Brazil.; [2] Veterinary Clinic Department – Veterinary Pathology Service – FMVZ-UNESP, Botucatu – SP, 18.618-000, Brazil.; [3] Octavio Bastos Veterinary Medicine College - UNIFEOB, São João da Boa Vista – SP, 18.370.000, Brazil.
goiozopfi@cnpgc.embrapa.br

Testis are site of various diseases that can lead the animal to infertility or low fertility, bringing economic losses to brazilian beef production. Testicular degeneration, hypoplasia, orchitis, circulatory alterations and neoplasias are the most frequent testicular changes. 159 testicles from 80 Nelore beef cattle, 26 month old, breeding in extensive conditions in andropogon´s pasture end supplemented with mineral salt, were evaluated gross and histopathologically. Scrotal perimeter (SP) was measure in its great diameter. After orchiectomy, length and width were measure from each testicle. For histopathology, the testicles were fixed in Bowin's solution and stained for HE and PAS. Among the diagnosis, degeneration was the most frequent, occurring in 65% of the animals. Orchitis (41.3%), circulatory changes (16.25%) and testicular hipoplasia (15%) account for the rest of the lesions. The scrotal perimeter from animals with testicular degeneration (26.58 cm) and hipoplasia (27.00 cm) didn't differ from animals with normal testicles (27.07 cm) ($p<0.05$) by Tukey´s test. In orchitis, the scrotal perimeter was lower ($p<0.05$), due to tubular tissue substitution by connective tissue. SP measurement is not a method that can be applied to identify animals with discrete or moderate hipoplasia, showed the importance of the reproductive control of the herd, therefore animals with discrete hipoplasia testes don't have change in fertility. The results of that work will add national data about testicular changes since epidemiological testicular changes information is sparse in the literature, supplying subsidy to research under male bovine fertility.

EVALUATION OF TESTOSTERONE PLASMA LEVELS AND DIFFERENCES AMONG RECTAL, ESCROTAL AND INTRATESTICULAR TEMPERATURES AND NUMBER OF SWEAT AND SEBACEOUS GLANDS IN BUFFALOS' SCROTUM (*Bubalus bubalis*) OF TWO AGE GROUPS.

Satrapa, R.A.[1]; Diniz, E.G.[2]; Beletti, M.E.[2]; Eberhardt, B.G.[1]; Satrapa, R.[2]

[1]Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil.[2]Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil.
rsatrapa@yahoo.com.br

The increase in testosterone basal levels is due to the differentiation of Leydig cells in association with the germinative cells proliferation, and the animal depends directly on these phenomena to reach the puberal age (AMANN, Journal of Animal Science, v.57, p.380-403). For those animals that have extra-abdominal testis, testicular temperature is lower than the body temperature, which leads to an appropriate, crucial, thermic environment to normal spermatogenesis (MACDONALD, Theor. Biol., v. 145, n. 4, p. 430). In cattle (*Bos taurus*), the importance of the sweat glands in testicular thermoregulation is known by the fact of, under high environmental temperature, scrotum produces five times more sweat than skin in other areas of body (ROBERTSHAW, Aust. J. Agric. Res., v.31, p.401-407). The purpose of this research was to evaluate testosterone plasma levels, differences among the rectal (RT), scrotal (ST) and intratesticular (IT) temperatures and differences between number of sweat (SG) and sebaceous (SbG) glands in buffalos' scrotum. 52 Murrah buffaloes were separated into animals of 12 to 15 months of age (Group I) and of 18 to 24 months (Group II), each group with equal number of animals. Testosterone concentration was assessed by radioimunoassay using plasma obtained after centrifugation of peripheral blood taken from each animal. RT were determined by clinical thermometer and ST and IT by digital thermometer. Sweat glands number was histologically determined in slides prepared with little slices of the distal scrotum skin collected from each animal and by means of optical microscopy. Data were assessed by student test. The average of testosterone plasma concentrations in Groups 1 and 2 (322 and 267 ng/dL, respectively) were statistically different ($p<0.05$). RT, ST and IT average of Group I were 38.6°C, 35.6°C and 36.9°C, respectively and in Group II, 39.1°C, 36.0ºC and 37.7°C, respectively. Inside each age group, RT, ST and IT were different ($p<0.05$) and also among them, except for ST, which was not different between the two age groups ($p=0.25$). Sweat and sebaceous glands average number in Group 1 (1.14 e 1.11, respectively) were different ($p<0.05$) from those of Group 2 (1.42 and 1.31, respectively). It was concluded that, as the animal reaches the reproductive age, responsiveness of hypothalamo-pituitary-gonadal axis increases to testosterone. Besides, scrotal and intratesticular temperatures are lower than that from rectum and the number of sweat and sebaceous glands increase in scrotum of those animals following the age.

IGFBP-3 LEVEL ASSESSMENT IN BULL SEMINAL PLASMA: TECHNIQUE VALIDATION

Viau, P.[1]; Oliveira, C.A.[1]; Souza, L.W.O.[1,2]; Arruda, R.P.[1,2]

[1] Departamento de Reprodução Animal – FMVZ/USP, São Paulo-SP, 05508-000, Brasil
[2] Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP Pirassununga-SP, 13630-000, Brasil. arrudarp@usp

Insulin-like Growth Factor Bindign Protein-3 (IGFBP3) is a 43 kDa protein found in several body fluids as a part of a complex mechanism that controls the availability of Insulin-like Growth Factors. The current knowledge points to a reproductive system modulation by locally produced growht factors and proteins, besides the hypothalamic-hypophiseal-gonadal axis. Insulin-like Growth Factor-I (IGF1) and its receptor was detected in Leydig cells and the secretion of IGFBP3 was found to be secreted by Sertoli cell in culture. This binding protein is also produced by prostate epithelial and stromal cells and either enhances or inhibits the effects of IGF1 on prostate epithelial cells, with the most of IGFBP3 detected in seminal fluid being in small fragments (≤ 16 kDa). The aim of this study was to validate de IGFBP-3 dosage in mature bull seminal plasma, using a solid-phase immunoradiometric (IRMA) assay, employing a commercial diagnostic kit (ACTIVE® DSL-5600, Diagnostic System Laboratories, Webster, Texas, USA) developed to quantitative evaluation of IGFBP-3 in human serum. The kit uses the protein labeled with ^{125}I as a tracer. Weekly semen samples were collected form seven purebred Simmental bulls aging from 38.8 to 41.5 months by electroejaculation (Eletrojet®, Eletrovet, Brasil). Seminal plasma were obtained by two-step centrifugation (500 g / 15 minutes and 6.000 g / 15 minutes at 4 ºC) and stored at -20 °C until lab manipulation. Protein level assessment was proceeded according to manufacturer protocol. Quality control of IRMA was done through analysis of inter and intra-assay coefficient of variation; 9.01% and 1.16%, respectively. Minimum sensibility detected was 0.04ng/mL. For the validation protocol was used a pool of samples with low protein concentration (0.74ng/mL). To the pool sample were added known values of IGFBP-3 in order to approximate to the standards available in the commercial kit (2.0, 5.0, 20.0, 50.0, and 100.0 ng/mL of IGFBP-3). To verify the parallelism in employed method of validation, was performed simple regression analysis and the correlation index was r=1.0 ($Y=0.037 + 0.878 * X$; $R^2=0.999$). Sixteen samples were assayed with de kit standards and the a curve was performed with the biological matrix. Correlation index obtained was r=0.99 ($Y = -0.145 + 1.122 *X$; $R^2=0.987$). Thus, it was concluded that was observed the parallelism between the standard curve of the kit and the curve obtained from the serial dilution of the samples pool. It was demonstrated mathematically that there were no interferences of the matrix in the antigen-antibody binding reaction, and it was validated the use of the commercial kit DSL-5600 (Diagnostic System Laboratories, Webster, Texas, USA) to quantify IGFBP-3 in bull seminal plasma.

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IMMUNOASSAY OF INSULIN-LIKE GROWTH FACTOR-I IN BULL SEMINAL PLASMA

Oliveira, C.A.[1]; Viau, P.[1]; Felippe, E.C.G.[1]; Souza, L.W.O.[2]; Arruda, R.P.[2]; Negrão, J.A.[3]

[1] Departamento de Reprodução Animal – FMVZ/USP, São Paulo-SP, 05508-000, Brasil. [2] Departamento de Reprodução Animal – FMVZ/USP, Pirassununga-SP, 13630-000, Brasil. [3] Departamento de Ciências Básicas – FZEA/USP, Pirassununga-SP, 13630-000, Brasil.
arrudarp@usp.br

The presence of IGF1 in testicular tissues and secretions was detected in rats, swine, and horses and humans. The concentration of IGF1 in bovine seminal plasma was found to be similar to that in human seminal plasma. This growth factor is pointed as possible modulator of testicular function through paracrine and autocrine mechanisms. Many researchers consider the radioimmunoassay (RIA) as the most practical and adequate way to quantify IGF. The objective of this study was to quantify IGF1 in bull seminal plasma using the radioimmunoassay (RIA) technique in solid-phase by way of a diagnostic commercial kit (IRMA ACTIVE® DSL-5600, Diagnostic System Laboratories, Webster, Texas, USA) developed to assess quantity IGF1 in human serum. The kit uses the growth factor labeled with ^{125}I as a tracer. Weekly semen samples were collected form seven purebred Simmental bulls aging from 38.8 to 41.5 months by electroejaculation (Eletrogen®, Santa Lydia, Presidente Prudente, Brasil). Seminal plasma were obtained by two-step centrifugation (500 g / 15 minutos e 6.000 g / 15 minutos à 4 ºC) and stored at -20 °C until lab maniupulation. Hormone dosage was proceeded according to manufacturer protocol, and the commercial kit was validated for use in bull seminal plasma by parallelism method ($Y = 3,045 + 0,701 * X$; $R^2 = 0,99$). Quality parameters obtained were below of 9.09 for intra-assay coefficient of variation and 4.17 for inter-assay coefficient of variation. An extraction procedure of samples was performed using ethanol/acid solution, when IGF1 was separated for binding proteins. Results are expressed in ng/mL and data descritive statistics were conducted using the Statistical Analysis System (SAS Institute Inc., 1985). Seminal plasma concentration of IGF1 showed high variation between bulls and between consecutive ejaculations. Mean concentrations of IGF1 (± SD) in seminal samples of the bulls ranged from 22.2 ± 7.1 to 396.1 ± 116.2 ng/mL. Overall mean concentration was 174.2 ± 164.1 ng/mL. Along the seven weeks trial the mean concentration was 174.2 ng/mL, ranging from 114.2 to 218.9 ng/mL. Between bulls variations were higher than sampling variations in the same Bull and similar to previous reports. As seminal IGF1 is produced mainly in the testis, it is reasonable to admit that variation between collections be low. It was concluded that assay kit for IGF1 is a satisfactory technique in quantifying the growth factor in seminal plasma.

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NON-EXTRACTIVE IMMUNOASSAY FOR BOVINE SEMINAL PLASMA TESTOSTERONE

Oliveira, C.A.[1]; Viau, P.[1]; Felippe, E.C.G.[1]; Souza, L.W.O.[2]; Arruda, R.P.[2]; Negrão, J.A.[3]

[1] Departamento de Reprodução Animal – FMVZ/USP, São Paulo-SP, 05508-000, Brasil. [2] Departamento de Reprodução Animal – FMVZ/USP, Pirassununga-SP, 13630-000, Brasil. [3] Departamento de Ciências Básicas – FZEA/USP, Pirassununga-SP, 13630-000, Brasil.
arrudarp@usp.br

Testosterone is almost exclusively produced in testis and its concentration in seminal plasma is correlated to number of spermatic cells, number of motile cells and other sperm characteristics. Quantification of testosterone has been conducted in several kinds of samples from many species through radioimmunoassay (RIA). Testosterone levels were determined by RIA after extraction procedure in blood serum, interstitial testicular fluid and testicular cytosol of rats; incubation media of hamster testicular tissue using RIA kit; blood and seminal plasma of bulls by enzymatic immunoassay (EIA) after extraction using tertiary butylmethylether. The aim of this work was to assess testosterone levels in seminal plasma of mature bulls using a RIA technique that employs a solid-phase commercial kit (ACTIVE® DSL 4000 RIA, Diagnostic System Laboratories, Webster, Texas, USA) developed to quantitative evaluation of testosterone in human serum. This commercial kit uses the hormone labeled with ^{125}I as a tracer. Seven weekly collection of semen samples, were obtained from eight purebred Simmental bulls aging from 33 to 41 months by electroejaculation (Eletrogen®, Santa Lydia, Presidente Prudente, Brazil). Seminal plasma was separated by two-step centrifugation (500 g / 15 minutes e 6.000 g / 15 minutes at 4 ºC) and stored at -20 °C until the lab manipulation. Hormonal quantification was proceed according to the manufactures protocol, the kit was validate for use in bull seminal plasma by parallelism method ($Y = 5,47 + 1,073 * R^2 = 0,98$), and inter and intra-assay coefficients were below to 11.57 and 4.51, respectively. Results are expressed in ng/mL and the descriptive statistics were proceeded using the Statistical Analysis System (SAS Institute Inc., 1985). Seminal plasma concentration of T varied between bulls and between consecutive ejaculations. Mean concentrations of T (± SD) in seminal samples of the seven bulls ranged from 0.16 ± 0.30 to 1.40 ± 0.74 ng/mL along seven weekly collections. Overall mean concentration was 0.60 ± 0.65 ng/mL. Along the seven sampling weeks mean concentration of the eight bulls ranged from 0.32 ± 0.29 e 0.75 ± 0.67 ng/mL. It was expected higher levels of seminal testosterone since the hormone is produced in the testicles and previous reports pointed higher values. Although, breed and environmental conditions during collection period must be considered as well as age differences between animals used in different trials. Another important factor affecting hormone dosage are binding proteins in seminal plasma that can reduce hormone quantity available for antigen-antibody of the RIA when not removed. Thus, RIA without extraction process can be only used when is intended to do comparisons and the objective is not to determine the total quantity of testosterone in seminal plasma.

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CHARACTERIZATION OF BINDING-HEPARIN PROTEINS IN THE SEMINAL PLASMA AND SPERMATOZOIDS IN NELORE BULLS

Fernandes, C. E.[1]; Souza, F.F.[1]; Neto, J.S.[2]; Ribolla, P.E.[2]; Feliciano Silva, A.E.D.[1]

[1] Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. [2]Instituto Biológico, UNESP, Campus de Botucatu, SP. cesfe@nin.ufms.br

Of the present proteins in the bovine semen, many present properties related to the lipoproteins of high density (HDL), such as the calmodulin and the heparin. These proteins have been recognized with BSPs (Sperm Binding Proteins) and whose primordial functions involve the relationship to the phospholipids of the sperm membranes, participation in the capacitation process and modulation of the acrosomal reaction. The objective of this study was to separate heparin-binding proteins in Nelore bulls using heparin affinity chromatography. Samples (4 mL) of one ejaculated of four bulls were separate for centrifugation (10.000 g/30 min) in two fractions: seminal plasma and spermatozoids. Aliquots of 100 L of each fraction were balanced in citrate-phosphate 50 mM, pH 8,0 and submitted the a flow from 650 to 1.000 mL/min. in chromatography system for heparin-binding (Heparin-Sepharose). At the end of the chromatography, 20 µL of the representative fractions of the major absorbance of the heparin-binding proteins after gradient of NaCl (1M), one-dimensional electrophoresis were conduced (SDS-PAGE, 12.5%, with molecular weight standard between 10 and 250 kDa). The resulting gels were stain with comassie blue and digitally analyzed. In the seminal plasma they were found proteins of 65.0, 58.0, 54.3, 38.6, 25.0 22.0 17, 4, 15.0 and 10.0 kDa (close values of 100 mABS), in the spermatozoids they were found proteins of 65.0, 55.3 47.6, 15.1, and 12.9. kDa (close to 40 mABS). Those results suggest that several proteins, besides the already described in *Bos taurus* in the different studied fractions, they can be present in the semen of Nelore bulls and therefore are used as indicators associated to the fertility.

EFFECTS OF SOMATOTROPIN ON THE SEMINAL CHARACTERISTICS OF *Bos taurus taurus* UNDER TESTICULAR HEAT STRESS CONDITIONS

Souza, L.W.O.[1]; Garcia, A.R.[1]; Celeghini, E.C.C.[1]; Andrade, A.F.C.[1]; Raphael, C. F.[1]; Nascimento, J.[1]; Rodrigues, P.H.M.[2]; Arruda, R.P.[1]

[1] Centro de Biotecnologia em Reprodução Animal - VRA/FMVZ/USP, Pirassununga-SP, 13630-000, Brasil, arrudarp@usp.br [2] Departamento de Nutrição e Produção Animal - FMVZ/USP, Pirassununga-SP, 13630-000, Brasil.

Growth Hormone (GH) exerts its effects through Insulin-like Growth Factor I (IGF1), a powerful mitogenic agent important to germ cell development and sperm cell maintenance. GH administration to bulls promotes morphological abnormalities reduction and motility improvement, increasing fertility. A randomly experimental block design used sixteen purebred Simmental bulls in a 2x2 factorial treatment arrangement (0 and 96 hours of scrotal insulation; 0 and 1.2 mg bST/kg LW IM – five applications each two weeks). Samples were weekly collected by electroejaculation (Eletrogen®, Santa Lydia, Presidente Prudente, Brasil), and assessed in regard to fresh semen characteristics (mass movement, motility and vigor) and cell morphology by phase contrast optic microscopy. A semen fraction was processed in order to obtain seminal plasma by two-step centrifugation (500 g / 15 minutes e 6.000 g / 15 min minutes a 4 ºC). Testosterone levels determinations were done by use of a RIA commercial kit ACTIVE® DSL4000 and IGF1 was assessed by use of a IRMA commercial kit ACTIVE® DSL5600 (Diagnostic System Laboratories, Webster, Texas, USA). Variance analysis employed the PROC GLM of the software Statistical Analysis System (SAS Institute Inc., 1985), adding the factor repeated measures, referring to the various sampling moments. Probabilities of interactions with time were determined by Greenhouse-Geisse test. Significance level adopted was 5%. In the whole period of semen collections, insulation caused increase ($P<0.05$) in the average of acrossome alterations occurrence (0.6%), midpiece defects (1.1%), head abnormalities (18.8%), tail defects (7.4%), and overall abnormalities (34.8%). Sperm motility was reduced ($P<0.05$) by insulation (50.4% average). Such reduction was proportional to overall sperm defects and can be result of morphological abnormalities. Seminal plasma testosterone (0.60 ng/mL) and IGF1 (150 ng/mL) were not influenced ($P>0.05$) by testicular insulation. It is reasonable to admit that bST did not act at testicular level since the majority of IGF1 comes from local production e there were no level increasing of this factor in the samples. Bovine somatotropin did not affect ($P>0.05$) sperm characteristics or seminal testosterone and IGF1.

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EFFECTS OF BOVINE RECOMBINANT SOMATOTROPIN ON CHROMATIN STRUCTURE OF BULL SPERMATOZOA

Garcia, A.R.[1,2]; Souza, L.W.O.[1]; Celeghini, E.C.C.[1]; Rodrigues, P.H.M.[1]; Andrade, A.F.C.[1]; Raphael, C.F.[1]; Nascimento, J.[1]; Arruda, R.P.[1]

[1] Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP, Pirassununga-SP, 13630-000, Brasil, arrudarp@usp.br; [2] Faculdade de Medicina Veterinária – CESUMAR, Maringá-PR, 87050-390, Brasil.

Somatotropin is fundamental for body growth and development. Its usage could improve fertilization rates by acting on spermatic characteristics or favors spermatic metabolism. The objectives of this study were: 1) Evaluate if testicular degeneration induced by heat stress is capable to damage the structure of sperm chromatin; and 2) Study the effect of exogenous applications of recombinant bovine somatotropin on the number of cells with fragmented chromatin in bulls with testicular degeneration. The experimental design was in block, using repeated measure and with 2x2 factorial treatment arrangement. Sixteen Simmental (*Bos taurus taurus*) 34 ± 10 months bulls with reproductive capacity were used as sperm donors. All animals were maintained in the same environmental condition and semen was collected once a week by electroejaculation during 22 weeks, with a total of 352 ejaculates. On week 5, eight animals were submitted to testicular insulation (Group INSUL, n=4 and Group IbST, n=4) and eight were maintained intact (Group CONT, n=4 and Group bST, n=4). During weeks 10, 12, 14, 16, and 18, animals from groups bST and IbST received subcutaneous applications of bovine recombinant somatotropin (Lactotropin®, Elanco, São Paulo, Brazil; 1,2 mg/kg PV), while the other animals received (groups CONT and INSUL) placebo. Chromatin fragmentation was evaluated by the orange acridine dye technique using epifluorescence microscopy with an amplification of 1000x (excitation: 460-570 nm and emission: 460-610 nm). 500 spermatozoa were evaluated in each sample and were classified according to method described by Tejada and others (Fertility and Sterility, v. 42, p. 87-91, 1984). Data were submitted to ANOVA, using the general proceedings of the *SAS*. Immediately before the beginning of testicular insulation, the average number of spermatozoids with fragmented chromatin were 0.35 ± 0.29% (CONT), 0.90 ± 0.71% (bST), 0.10 ± 0.10% (INSUL), and 0.40 ± 0.12% (IbST). There was time influence (P=0.0001), and time X insulation (P=0.0001) effects on the results of DNA fragmentation. When testicular insulation was implanted, an increase in the number of cells with fragmented DNA was observed in INSUL and IbST bulls after the sixth week. The peak of abnormality occurred at the ninth week: 0.55 ± 0.25% (CONT), 0.45 ± 0.21% (bST), 9.95 ± 4.04% (INSUL) and 10.63 ± 2.78% (IbST). Treatment with bovine recombinant somatotropin did not influence the expression of spermatozoids with fragmented DNA, and in CONT and bST animals the averages were maintained below 1% during weeks 10 to 22. On the other hand, there was reduction in the number of DNA fragmented cells in INSUL and IbST animals. Once this reduction was slower for IbST animals, significant effect for the treatment with bovine recombinant somatotropin was not observed (P=0.8807). After 22 weeks, the averages of fragmented DNA spermatozoids were 0.56 ± 0.11% (CONT), 0.82 ± 0.14% (bST), 1.48 ± 0.32% (INSUL), and 3.44 ± 0.70% (IbST). Consequently, testicular insulation is capable to induce a transitory increase in the number of spermatozoa with fragmented chromatin, while bovine recombinant somatotropin did not accelerate the reestablishment of the normality of spermatic chromatin structure, if used after the installation of testicular degeneration.
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USE OF CMXROS AND JC-1 ON MITOCHONDRIAL FUNCTION EVALUATION, ASSOCIATED TO FLUORESCENT PROBES TO PLASMATIC AND ACROSOMAL MEMBRANES EVALUATION IN BOVINE SPERMATOZOA

Celeghini, E.C.C.[1]; Nascimento, J.[1]; Andrade, A.F.C.[1]; Raphael, C.F.[1]; Souza, L.W.O.[1]; Arruda, R.P.[1]

Centro de Biotecnologia em Reprodução Animal – VRA/FMVZ/USP
13630-090, Pirassununga-SP, Brasil. arrudarp@usp.br

The fluorescent probes association to simultaneous evaluation of the plasmatic and acrosomal membranes integrity and the mitochondrial function of the bovine spermatozoa, have been utilizing to development a laboratorial techniques to predict with more efficiently the fertilizing ability of bovine semen. The use of the MitoTracker red® or CMXRos and of the 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), fluorescent probes to mitochondrial function, was tested and validated in association with others fluorescent probes, such as propidium iodide (PI) and Hoechst 33342 (H342) to identify integrity of the plasmatic membrane and *Pisum sativum* agglutinin conjugated to fluorescein isotiocionate (FITC-PSA), to verify intact acrosome. To validation of these techniques were utilizing three semen collects (Eletrogen®, Santa Lydia, Presidente Prudente, Brazil) from four Simental bulls (n=12). Were used ejaculates that showed motility ≥80%, vigour ≥3 and abnormal morphology ≤10%. Semen was diluted in TALP medium ($25x10^6$ spermatozoa/mL) and split into two aliquots, one hold viable, and the other was submitted to flash frozen in liquid nitrogen and slowly thawed in three continuous cycles, to induce damage in cellular membranes and to perturb mitochondrial function. Three treatments were prepared with the following fixed ratios of fresh semen: flash frozen semen: 100:0 (T100), 50:50 (T50), and 0:100 (T0). A 150-μL aliquot from diluted semen was added 2 μL of H342 (40 μg/mL), 3 μL of PI (0.5 mg/mL), 50 μL of FITC-PSA (100 μg/mL), and 0.5 μL of CMXRos (500 μM) or 2 μL of JC-1 (153 μM). The samples were incubated at 38.5°C/8 min, in the dark. After the incubation, humid preparations were made and the reading were performed immediately on epifluorescent microscopy in the filters with 460-570 and 365nm excitation and 460-610 and 420nm emission, counting 200 cells. According to fluorescence emitted by each probe utilized eight class of cells were established. The data obtained of plasmatic and acrosomal membranes integrity and mitochondrial function (dependent variable) in the treatments T100, T50 and T0 (independent variable) were submitted to regression analysis. To association PI/H342/FITC-PSA and CMXRos, the equations obtained were: Y=1.64+0.82X (R2=0.93), to plasmatic membrane integrity, Y=3.50+0.84X (R2=0.92), to intact acrosome, and Y=1.50+0.80X (R2=0.93), to mitochondrial function. To the other association PI/H342/FITC-PSA and JC-1 were obtained the following equations: Y=0.78+0.92X (R2=0.92), to plasmatic membrane integrity, Y=3.82+0.81X (R2=0.91); to intact acrosome and Y=0.06+0.78X (R2=0.93) to potencial of mitochondrial membrane. Based in the equations and the higth determination coeficient in the both techniques is possible to conclude that they are efficient to simultaneous evaluations of the plasmatic, and acrosomal membranes integrity and mitochondrial function in bovine spermatozoa. However, a advantage from JC-1 on CMXRos was observed, once JC-1 separates two populations of cells by color code, showing mitochondria with high potential of membrane, dyed in red, and low potential of membrane, dyed in green.

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ULTRAESTRUCTURAL AND DNA FRAGMENTATION ASSESSMENT OF BOVINE SPERMATOZOA CONSERVATED BY DIFFERENT TREATMENTS OF SPERM DRIED.

Martins, C.F.[1,2]; Báo, S.N.[2]; Rumpf, R.[1]

[1] - Embrapa Recursos Genéticos e Biotecnologia [2] - Universidade de Brasília
rodolfo@cenargen.embrapa.br

Freeze drying or lyophilization is a procedure developed to preserving biological materials, pharmaceuticals and solvent-impregnated materials. This procedure has been devised to achieve preservation by restricting the active water in the biological systems for sublimation of the ice. Recently, freeze-drying has been applied to preserve mammalian spermatozoa, representing an alternative tool for conservation of genetic material. This way, the objective of this work was verify the effect of sperm freeze-drying with different treatments on the structure of bovine spermatozoa, evaluate for eletron microscopy, acridine orange stain and TUNEL technique. Spermatozoa of three bulls were collected by eletroejaculation and mixed with three different solution: (T 1) - TCM hank's with 10% of SFB; (T 2) - TCM hank's with 10% of SFB and trehalose 0,2 M; (T 3) - Solution of EGTA, consisted of 10mM of tris-HCl and 50mM of EGTA [ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid]. As control was used frozen semen conventionally. The samples were frozen in liquid nitrogen and quickly placed in the freeze-drying system, where were lyophilizated about 12-16 hours. To verify the eventual effects of sperm dried were evaluate the main structures involved in the fertilization. For this was used ultrastructural evaluation by eletron microscopy and DNA fragmentation by acridine orange stain and TUNEL technique. In all the treatments the spermatozoa lost the motility, however, there was a small lose of tails (separation of the head). The plasma membrane suffered larger alterations in the T2 and T3, but in the T1 only small undulations was observed. The acrossomal structure and the mitochondria were intact in most of the spermatozoa in both treatments. The microtubules suffered completes disarrange in the T2 and T3, and maintained normal in the T1. The sperm chromatin integrity was 95%, 98%, 100% and 100%, respectively for T1, T2, T3 and control. For the TUNNEL technique was observed 14%, 5%, 2% and 1% of DNA fragmentation, respectively for the treatments T1, T2, T3 and control. These results indicate that sperm freeze drying is aggressive, turning immobile the spermatozoa, however, the nucleus seems well protected by the treatments T2 and T3. The fact of the nucleus to stay intact it still represents fertilization possibility, when are injected into oocyte cytoplasm by intracytoplasmic sperm injection technique.

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EFFECT OF PERCOLL GRADIENT ON THE ACROSOMAL INTEGRITY AND SPERM CHROMATIN FRAGMENTATION IN BULLS WITH ABNORMAL SPERMIOGENESIS

Fernandes, C.E.[1]; Feliciano Silva, A.E.D.[1]; Dode, M.A.N.[2]

[1] Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. [2]Cenargen- Embrapa, Brasília, DF, Brasil. cesfe@nin.ufms.br

The Percoll gradient (PG) it is one of the most used methods for sperm selection in the in vitro fertilization (IVF). Your advantages include separating spermatozoids of the other cells, bacteria and undesirable particles. Studies have been showing that the sperm population selected by that method it presents superior characteristics in kinetics and sperm morphology. However, in the last few years the effect of some morphological characteristics, especially those related with sperm nucleus integrity, have been more investigated. Those have important role in the low efficiency of bulls in the in vitro embryos production. The objective of this study was to evaluate the effect of sperm selection, by Percoll gradient on acrosomal integrity and nuclear fragmentation in bulls with abnormal spermiogenesis. Four Nelore bulls with age between 2 and 3 years were subjected to 5 days scrotal insulation period. The semen was collected by electro ejaculation and frozen (Tris-lactose, glycerol and egg yolk extender) before (P0) and after remove the testicular insult in the 7 (P7), 14 (P14) and 21 (P21) days. Three samples were thawed of each bull in each period being evaluated the acrosomal integrity (AcI) by tryplan –blue giemsa stain (200 cells/1000x/bright-field) and sperm chromatin fragmentation (SCF) by Fuelgen stain (500 cells/1000x/phase-contrast) pre and post PG (2 mL of Percoll 45 and 2 mL of percoll 90%, centrifuged at 700 x g for 20 minutes at 30 ^{o}C). The results showed that there was no interaction between the experimental periods and PG for AcI. That variable was higher ($p<0.05$) just post PG in the P7 (65.2±6.8), P14 (67.6±0.95) and P21 (72.8±12.0) periods in relation to P0 (47.8±4.5). SCF was superior ($p<0.05$) post PG in P21 (32.4 ±3.5) in relation to pre PG (22.4±3.4). It is concluded with those observations that the gradient of Percoll can favor the selection of spermatozoids with higher integrity acrossomal, however, it is not capable to retain spermatozoids with high percentile of nuclear chromatin fragmentation.

EFFECTS OF SEMINAL PLASMA REMOVAL ON BOVINE FROZEN SEMEN

Alberti, K.[1]; Papa, F.O.[1]; Siqueira Filho, E.R.[1]; Melo, C.M.[1];
Dell'Aqua Jr., J.A.[1]

[1]Departament of Animal Reproduction and Veterinary Radiology, FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. karinaalberti04@yahoo.com.br

Bovine frozen semen has been successfully used for decades in the whole world. Several reproduction biotechniques have been developed and improved in this period. Nowadays, with the distinctive position of cattle raising in the country and the search for genetic improvement and economic income of raisings, innovations in semen freezing techniques are necessary. The seminal plasma removal is a very common technique routinely used in horse semen cryopreservation, being essential and necessary for the success of freezing. In rams, was showed significant improvement in post-freezing seminal parameters, after seminal plasma removal (Oba et al., International Congress of Animal Reproduction, Brazil, v.2, p.481, 2004). The present work aimed to test a new methodology for bovine semen freezing, by introducing centrifugation for seminal plasma removal. Forty ejaculates from bulls, able for reproduction and kept in pasture, were collected. Ejaculate was divided into six parts, so that three of them were diluted with TRIS, Glycine-Yolk and Botu-Bov® extenders and frozen by using the conventional protocols and the other three parts, one of each extender, were centrifuged with the fraction without cryoprotector for seminal plasma removal. After centrifugation, supernatant was discharged and pellets were resuspended again on the referred extenders. Both straws used in the conventional protocol and the ones of the group tested were cooled in refrigerators under 5°C for four hours, frozen in liquid nitrogen and kept in storage at –196°C. After thawing semen was submitted at CASA system, for measurement of spermatic parameters: Total Motility (TM), Progressive Motility (PM) and spermatozoa with rapid movements (RAPID). The membrane integrity (MI) was evaluated using fluorescent probes. Comparing results of spermatic parameters from the group whose seminal plasma was removed with the conventional protocols, it was observed a superiority of samples centrifuged, regardless of extender used. Averages (MT/MP/RAPID/IM) presented by extender Botu-Bov® using or not centrifugation were 66.17^{a} / 50.97^{a} / 57.60^{a} / 35.65^{a} and 58.57^{b} / 43.22^{b} / 48.37^{b} / 30.12^{b}; for extender Glycine-yolk values were 59.07^{b} / 44.00^{b} / 48.89^{b} / 28.72^{b} and 53.12^{c} / 38.82^{c} / 43.65^{b} / 26.37^{bc} and with extender TRIS, values were 54.17^{c} / 36.10^{c} / 48.45^{c} / 28.67^{b} and 46.92^{d} / 31.95^{d} / 38.79^{d} / 25.90^{c}, respectively. Results above show average improvement of 12.6% (MT), 14% (MP), 18.3% (RAPID) e 12% (IM) in the three extenders, after centrifugation. Data presented in the present study suggest that seminal plasma removal improves bovine semen freezability rate, showing that centrifugation is an important methodological innovation in the search for the progress of this technique and in the improvement of fertility rates in the different reproduction biotechnologies.

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BOTU-BOV® A NEW PROPOSAL FOR AN EXTENDER IN CRYOPRESERVATION OF BOVINE SEMEN

Alberti, K.[1]; Papa, F.O.[1]; Siqueira Filho, E.R.[1]; Melo, C.M.[1];
Dell'Aqua Jr., J.A.[1]

[1]Departament of Animal Reproduction and Veterinary Radiology, FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. karinaalberti04@yahoo.com.br

The modern bovine industry in the whole world is based on the use of artificial insemination (A.I.) with frozen semen (Aires, et al., Theriogenology, v.60, p.269-279, 2003). The development of appropriate techniques for semen preservation is one of the most important steps in the advance of animal reproduction in the different species and it has been achieved by means of the application of more and more modern biotechniques (Papa et al., Rev. Bras. Reprod. Anim., v.24, n.1, p.39-44, 2000). Although semen cryopreservation is a widely used biotechnology, it presents deleterious action during the freezing process and the purpose of new methodologies is to minimize these detrimental effects to semen. In this sense, the semen extender is essentially important for the preservation of spermatic cells, exposed to cryopreservation. Thus, obtaining extenders that promote higher viability of spermatozoons after thawing is indispensable. The present study aimed to test the efficiency of the extender Botu-Bov®, compared to extenders TRIS and Glycine-Yolk, in the cryopreservation of bovine semen by using the conventional freezing methodology. Semen from 40 bulls, 20 *Bos taurus taurus and* 20 *Bos taurus indicus*, in reproductive age and kept under pasture diet, was collected. Each sample was fractioned into three parts and diluted with extenders TRIS, Glycine-Yolk and Botu-Bov®. All samples were packed into 0,5 ml straws and cooled in automatic refrigerators under 5°C for stabilizing during four hours. After that, straws were exposed to liquid nitrogen steam (N_2) for twenty minutes and then immersed in N_2 and storage at –196°C. Post-thawing spermatic parameters were analyzed by CASA, where the percentages of total spermatic motility (TM), progressive spermatic motility (PM) and rapid spermatozoa (RAPID). The evaluation of plasmatic membrane integrity was performed by using fluorescent probes. The new extender presented statistically superior results by the Tukey test ($p<0,01$) in all spermatic parameters tested. Values (MT/MP/RAPID) found in the samples of Botu-Bov® were 58.57^a/ 43.22^a/ 48.37^a compared to Glycine-Yolk and TRIS, which obtained 53.12^b/ 38.82^b/ 43.65^b; 46.92^b/ 31.95^b/ 38.79^b, respectively. The result of the plasmatic membrane integrity analysis for the three extenders Botu-Bov®, Glycine-Yolk and TRIS were 30.12%^{a}, 26.30%^{b} and 25.90%^{b}. Partial data of artificial insemination using extender Botu-Bov® showed reproductive efficiency, projecting promising fertility rates. The results obtained in this experiment showed that Botu-Bov® extender promotes higher protection to spermatic cells in cryopreservation process, in relation to other extenders tested, resulting in higher post-thawing spermatic viability, as verified in laboratory tests. Thus, we can conclude that the extender tested confirms the initial objective of developing a new innovative extender for the techniques for bovine semen freezing.

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EFFECT OF BOTU-BOV® AND TRIS EXTENDERS ON SEMEN FREEZABILITY IN BULLS *Bos taurus taurus* & *Bos taurus indicus*

Alberti, K.[1]; Papa, F.O.[1]; Siqueira Filho, E.R.[1]; Melo, C.M.[1];
Dell'Aqua Jr., J.A.[1]

[1]Departament of Animal Reproduction and Veterinary Radiology, FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. karinaalberti04@yahoo.com.br

Beef cattle raising is one of the most important sectors in national economy nowadays. At the moment, bovine production in the country is based in two groups of animals from two distinct regions of the planet: the zebus, coming from the region of India, and the taurines, from the European continent. These two groups are very different from each other regarding their scientific, productive and reproductive characteristics. In Brazil, in order to attenuate such differences and obtain higher productivity in the herd, blood shock or industrial crossbreeding is commonly used, in which reproduction biotechniques such as artificial insemination (AI) are fundamental. For the AI advancement, semen quality to be used in this process is important; therefore, efficient extenders are necessary for the cryopreservation of semen from both genetic sources. The present study aimed to evaluate the efficiency of extender Botu-Bov® on freezability rates of zebus and taurines bulls. The experiment was carried out with 40 ejaculates from different bulls in reproductive activity, being 20 *Bos taurus taurus* and 20 *Bos taurus indicus*. Each ejaculate was fractionated into two parts, and each one of those diluted in Botu-Bov® and TRIS. Samples were packed and cooled in automatic refrigerator under 5°C for four hours and then frozen in liquid nitrogen. Post-freezing spermatic parameters were analyzed by CASA, determining total spermatic motility (TM), progressive spermatic motility (PM) and rapid spermatozoons (RAPID). The plasmatic membrane integrity (MI) was verifyied using carboxyfluorescein diacetate (CFD) and propidium iodide (PI), as fluorescent probes. Results found in the laboratory tests showed that Botu-Bov® extender was superior ($p<0,001$) regarding all spermatic parameters, when compared to TRIS. Botu-Bov® extender presented the following MT/MP/RAPID/IM averages: 57.45^a/ 42.50^a/ 48.80^a/ 29.40^a in zebus and 59.70^a/ 43.95^a/ 51.95^a/ 30.85^a, in taurines. The averages for TRIS extender were: 43.35^b/ 29.45^b/ 34.29^b/ 25.00^b e 50.50^c/ 34.45^c/ 43.30^c/ 26.80^b in zebus and taurines, respectively. In conclusion, there was no interaction between extenders and breed sources, with no differences between groups regarding post-freezing spermatic viability indexes. The data revealed that Botu-Bov® was superior in both breeds when compared to TRIS.

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EFFECTS OF CRYOPRESERVATION AND DILUENT ON BOVINE SEMEN WITH REGARD TO PLASMATIC AND ACROSOMAL MEMBRANES AND MITOCHONDRIAL FUNCTION

Celeghini, E.C.C.[1]; Andrade, A.F.C.[1]; Nascimento, J.[1]; Raphael, C.F.[1]; Bianconi, L.L.[1]; Rodrigues, P.H.M.[2]; Arruda, R.P.[1]

[1]Centro de Biotecnologia em Reprodução Animal - VRA/FMVZ/USP; [2]Departamento de Nutrição e Produção Animal - FMVZ/USP; 13630-090, Pirassununga-SP, Brasil. arrudarp@usp.br

Semen cryopreservation cause injury to sperm membranes, which reflect on reduced fertility. Theses injury are due medium temperature and osmolarity alterations, which induce morphologic changes in lipids organization and composition. However, different cryopreservation can to differ in preservation of several cellular compounds. The objectives techniques, such as the use of fluorescent probes, to sperm plasmatic and acrosomal membranes, and mitochondrial function evaluation can to provide information about cryoinjury. The objective of this experiment was to evaluation of cryopreservation effects on plasmatic, acrosomal and mitochondrial membranes of bovine sperm in front of two different diluents. Thus, seven semen collects from eight Simental bulls were performed. After the collect, the semen was simultaneous evaluated about plasmatic and acrosomal membrane integrity and mitochondrial function, by fluorecent probes association, using the following protocol: a 150-µL sample of diluted semen in TALP medium ($25x10^6$ spermatozoa/mL) was added of 3 µL of propidium iodide (PI, 0,5 mg/mL), 50 µL of *Pisum Sativum* agglutinin conjugated to fluorescein isotiocionate (FITC-PSA, 100 µg/mL) and 2 µL of 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, 153 µM). The sample was incubated (38.5°C/8 min) and the lecture was performed by epifluorescent microscopy (Nikon, Eclipse 80i) with filter (D/F/R, C58420) with 340-to-525 excitation ranges and 435-to-655 emission, counting 200 cells. After analysis, semen was split into two aliquots, one diluted in Bioxcell® (IMV Technologies, IVP do Brasil) diluent and other in Botu-Bov® (Biotech-Botucatu-Ltda/ME, Brazil) diluent, packaged in 0.5-mL straws ($50x10^6$ spermatozoa/straw) and submitted to automatized frozen system (TK-3000, TK Tecnologia em Congelação Ltda., Uberaba, Brazil). Two straws from each treatment was thawed and semen was evaluated again about plasmatic and acrosomal membrane integrity and mitochondrial function using the same technique reported above, with some modification (150-µL of semen diluted in TALP, $20x10^6$ spermatozoa/mL, + 3 µL of PI + 50 µL of FITC-PSA + 6 µL of JC-1). The statistical analysis was performed utilizing the Statistical Analysis System software (SAS Institute Inc., 1985); the data were submitted to analysis of variance and Tukey's test. The plasmatic membrane integrity percentual (78.06 ± 1.18%), intact acrosome (86.08 ± 0.92%) and mitochondrial function (76.88 ± 1.47%) of fresh semen declined ($P<0.05$), when compared to cryopreserved semen in both diluent. The cryopreserved semen with Botu-Bov® preserved better ($P<0.05$) the plasmatic membrane integrity (28.83 ± 1.11 x 18.16 ± 0.97%) and acrosomal (68.83 ± 0.99 x 55.26 ± 1.77%) and mitochondrial function (23.96 ± 1.06 x 16.96 ± 1.03%) than Bioxcell®. The plasmatic and acrosomal integrity membranes and mitochondrial function cell percentual on fresh semen (72.06 ± 1.43%) also decreased post-cryopreservation on Botu-Bov® (20.80 ± 1.03%) and Bioxcell® (11.19 ± 0.90%) diluents, been better preserved with Botu-Bov®. Conclusion, the semen cryopreservation cause great amount injury to spermatic membranes, which are less prominent when the semen was cryopreserved with Botu-Bov® than Bioxcell® diluents.

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EFFECTS OF CRYOPRESERVATION AND DILUENT ON MOTILITY OF BOVINE SEMEN

Celeghini, E.C.C.[1]; Andrade, A.F.C.[1]; Nascimento, J.[1]; Raphael, C.F.[1]; Rodrigues, P.H.M.[2] ; Arruda, R.P.[1]

[1]Centro de Biotecnologia em Reprodução Animal - VRA/FMVZ/USP; [2]Departamento de Nutrição e Produção Animal - FMVZ/USP; 13630-090, Pirassununga-SP, Brasil. arrudarp@usp.br

The injury caused during the semen cryopreservation are due medium temperature and osmolarity changes, which affect directly the sperm motility, have seen that this characteristic is crucial to sperm transport in the female reproductive tract, thus, can interfere in the fertility rate. The computer assisted semen analysis (CASA), have been used by tool to evaluate the sperm moving alterations more objectively. This experiment was elaborate with the objective to evaluate the cryopreservation and two diluents effects on sperm motility in bovine semen. Seven collects of semen were performed from eight Simental bull. After the collect, semen was evaluated about sperm motility evaluated subjectively, by visual estimative and objectively by CASA. The parameters evaluated by CASA were: total motile (%), progressive motility (%), path velocity (VAP, µm/s), progressive velocity (VSL, µm/s), track speed (VCL, µm/s), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). After analysis, the semen was split into two aliquots, one diluted in Bioxcell® diluent and other in Botu-Bov® diluent, packaged in 0.5-mL straws ($50x10^6$ spermatozoa/straw) and submitted to automatized frozen equipament (TK-3000, TK Tecnologia em Congelação Ltda., Uberaba, Brazil). Two straw from each treatment was thawed and the semen was evaluated again about the same characteristics of motility evaluated pre-cryopreservation. The statistical analysis was performed utilizing the Statistical Analysis System software (SAS Institute Inc., 1985); the data were submitted to analysis of variance and Tukey's test. The subjective motility percentual (73.13 ± 0.60%) and the motility parameters by CASA, such as total motile total (79.98 ± 1.36%), progressive motility (60.38 ± 1.98%), VAP (137.57 ± 1.87µm/s), VSL (121.12 ± 1.74µm/s) and VCL (193.97 ± 2.30µm/s) were higher to fresh semen than post-criopreserved semen with both diluent. The semen cryopreserved with Botu-Bov® preserved better subjectively motility (38.57 ± 1.66 x 25.09 ± 1.31%), total motile (40.13 ± 1.92 x 24.18 ± 1.44%) and progressive motility (20.86 ± 1.46 x 10.71 ± 0.98%) than Bioxcell®. While, Bioxcell® conserve higher VAP (92.45 ± 2.63 x 80.35 ± 1.37µm/s), VSL (81.57 ± 2.39 x 73.17 ± 1.30µm/s) and VCL (145.24 ± 4.11 x 113.81 ± 2.06µm/s) do que Botu-Bov®. The value of fresh semen BCF (36.22 ± 0.44Hz) decrease when compared to cryopreserved semen with Bioxcell® (33.95 ± 0.48Hz); however, increased when semen was cryopreserved with Botu-Bov® (38.48 ± 0.53Hz). The STR was higher in cryopreserved semen with Botu-Bov® (90.38 ± 0.38%) than with Bioxcell® (87.98 ± 0.43%) or fresh semen (86.96 ± 0.40%). The LIN was lower in cryopreserved semen with Bioxcell® (58.64 ± 0.76%) than with Botu-Bov® (66.07 ± 0.78%) or fresh semen (64.24 ± 0.69%). In conclusion, the semen cryopreservation promote sperm motility decrease and the use of diluent with different contents affect the motility and sperm velocity percentual distinctly in bovine semen.

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EFFECTS OF CRYOPRESERVATION USING DIFFERENT FREEZING TECHNIQUES AND CRYOPROTECTANTS ON PLASMATIC, ACROSOMAL AND MITOCHONDRIAL MEMBRANES OF BOVINE SPERMATOZOA

Arruda, R.P.[1]; Forero-Gonzalez, R.A.[1]; Celeghini, E.C.C.[1]; Raphael, C.F.[1]

[1]Centro de Biotecnologia em Reprodução Animal – VRA / FMVZ / USP, Pirassununga-SP, 13630-900, Brasil. arrudarp@usp.br

During cryopreservation spermatozoas undergo many physical-chemistry changes leading to different degrees of damage in their structure. Between the factors that have influence in freezing results, freezing curves and type of cryoprotectant used, deserve attention. This experiment was designed to compare the effects of cryopreservation of bovine spermatozoa using two freezing techniques ("conventional" and automated), with different curves and the use of three cryoprotectants (glycerol, ethyleneglycol and dimethylformamide) on the motility and integrity of spermatic membranes (plasmatic, acrosomal, and mitochondrial). Six collections were perfomed in five Simmental bulls. After collection (Eletrogen®, Santa Lydia, Presidente Prudente, Brazil), semen was evaluated to motility and concentration, and then diluted in TRIS-yolk prepared previously with each of following cryoprotectants: glycerol (7%), ethyleneglycol (7%) or dimethylformamide (3%). Half of the doses was frozen by a conventional technique (TC), straws were put on horizontal position on a grid and this in a 15L thermal box with six kilograms of ice for 90 minutes (cooling rate, -0.55°C/min), so the grid was transferred to another thermal box when straws stayed in nitrogen vapor (3 cm above liquid nitrogen) for 15 minutes (freezing rate, -19.1°C/min). The other half of straws were frozen by automated technique (TA) using a programmed machine (TK3000- TK Tecnologia em Congelação Ltda., Uberaba, Brasil) when straws were put in vertical position, with establishes curves of cooling (-0.23°C/min) and freezing (-15.5°C/min). After freezing, straws were plunged into liquid nitrogen and stored at -196°C, until the thawed at 37ºC for 30 seconds. Evaluations of motility were assessed using optic microscopy, and the integrity of plasmatic, acrosomal and mitochondrial membranes were evaluated using propidium iodide (PI), fluorescein conjugated *Pisum sativum* agglutinin (FITC-PSA) and MitoTracker Green FM (MITO), respectively, by fluorescence microscopy. Effects of freezing techniques and the different cryoprotectants in motility, integrity of plasmatic and acrosomal membranes and mitochondrial function were analyzed by analysis of variance using StatView® Program, (SAS Institute, Cary, USA). The means were compared by *Fisher*'s test. There were no significant differences between freezing techniques and no interaction among cryoprotectant and freezing techniques ($P>0.05$). There were differences between cryoprotectants ($P<0.05$). Mean percentage for motility with glycerol were 30.67±1.41 in TC and 30.50±1.06% in TA, ethyleneglycol 21.17±1.66 in TC and 21.67±1.13% in TA, and dimethylformamide 8.33±0.65 in TC and 9.17±0.72 % in TA. The percentage of spermatozoa with intact plasmatic and acrosomal membranes and with mitochondrial function to glycerol were 15.58±1.51 in TC and 16.68±1.31% in TA, ethyleneglycol 9.70±1.36 in TC and 10.38±1.30% in TA, and dimethylformamide 5.33±1.09 in TC and 5.57±0.88% in TA. In this experiment glycerol was the best cryoprotectant, although nearly 85% of spermatozoa showed some degree of injury in their membranes, evidencing that further studies are required to improve the results of cryopreservation of bovine semen.

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Abstracts - Session - B

IGF-I CONCENTRATIONS IN FOLLICLES FROM NELORE COWS

Melo, D.S.[1]; Leal, L.S.[1]; Gioso, M.M.[1]; Costa, I.B.[1]; Machado, M.S.[1]; Assaf, S.S.[1;]
Fernandes, C.B.[1]; Oba, E.[1]

[1 De]partment of Animal Reproduction and Veterinary Radiology– FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. danilassal@yahoo.com.br

The *in vitro* production of bovine embryos (IVP) is a reproductive biotechnology that contributes to the genetic growth and improvement of herds in Brazil. The oocyte growth inside a developing ovarian follicle is controled by numerous factors affecting oocyte competence and viability during *in vitro* development. Some of those factors are: follicular size, day of estrus, atretic condition and the influence of a dominant follicle. Oocytes obtained by ovum pick-up, which are submitted to *in vitro* production, are recovered from diferent stages of development and at a distinct phase of the estrous cycle. Progesterone, Estradiol, LH, FSH, and Growth Factors can potentially be affecting the oocyte competency during *in vitro* embryo development. Some of those factors (IGF-I, IGF-II, EGF, TGFα, TGFβ and others) were determined during recruitment, selection and growth phase of follicular development. In addition, their role on nuclear and cytoplasmatic maturation has been investigated in bovine oocytes. The objective of the present work was to evaluate the intra- follicular IGF-I (Insulin-like Growth Factor) concentrations in follicles with ≥6,5mm and <6,5mm in diameter obtained from Nelore cows. And upon this information, verify possible changes on its concentrations during a dominant phase from a follicular wave. Ovaries were recovered from 26 cows at slaughter house and transported in saline (NaCL 0,9%) at 36°C to the Endocrinology Lab (RARV) at the Department of Animal Reproduction and Veterinary Radiology, UNESP, Botucatu-SP. The ovaries were evaluated by ultrasound (Aloka 500, 5 MHz), to verify the presence and size of a dominant follicle. Follicles ≥ 6.5mm were considered as dominants (DF group), and their follicular fluid aspirated and stored individually. All the remaining subordinate follicles < 6.5 mm obtained from the same animal were aspirated and follicular fluid was stored within a pool (SF group). IGF-I was determined by a commercial kit (DSL-Diagnostic Systems Laboratories, Corporate Headquarters, 445 Medical Center Blvd, Webster, Texas) and quantified by Radioimmunoassay, Cobra II. The data were analyzed by ANOVA using SAEG. The DF group showed higher concentrations of IGF-I (184.61 ± 19.31 ng/mL, P=0.009) than the SF group (113.79 ± 17.23 ng/mL). These results indicate that in Nelore cows after deviation, the dominant follicle can influence the IGF-I concentrations from the remaining follicles. In addition, low concentrations of IGF-I and other Growth Factors, followed by hormonal changes occurring at the dominant phase can possibly induce follicular atresia and alter the oocyte competency, which will affect number and quality of *in vitro* produced embryos. However, both experimental groups may have atretic and subordinate follicles in variable proportions, wich migth influence the results obtained.

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FIBROBLASTIC GROWTH FACTOR 10 (FGF-10) GENE EXPRESSION DURING BOVINE LUTEAL DEVELOPMENT

Giometti, I.C.[1]; Castilho, A.C.S.[1]; Andrade, P.B.[1]; Costa, I. B.[1]; Machado, M.F.[1]; Price, C.A.[2]; Papa, P.C.[3]; Buratini Jr., J.[1]

[1]Department of Physiology, IB, UNESP-Botucatu. [2]Centre de Recherche en Reproducion Animale, University of Montreal, Canada. [3]Department of Surgery, FMVZ, USP.
inesgiometti@yahoo.com.br

The corpus luteum (CL) is formed after ovulation and secretes progesterone, which is fundamental for pregnancy initiation and maintenance. Angiogenesis and cellular proliferation and differentiation are important for CL formation. There are evidences indicating the involvement of growth factors such as FGFs in these processes. FGF-1, FGF-2 and FGF-7 gene expression was detected in the bovine CL. FGF-10 mediates paracrine interactions between mesenchimal and epithelial cells, regulating cellular proliferation and differentiation. However, the involvement of FGF-10 in the control of luteal development has not been addressed yet. Thus, the objective of this study was to investigate FGF-10 gene expression in the bovine CL. Ovaries were obtained in a local abattoir, 10 CLs from each of the four development stages (stage 1=corpus hemorragicum, stage 2=developing CL, stage 3=mature CL and stage 4=luteolytic CL) were selected, and 50-100mg of luteal tissue were submitted to RNA extraction (Trizol; Invitrogen®). FGF-10 gene expression was examined by semiquantitive RT-PCR using bovine-specific primers. Semiquantitative RT-PCR was validated by choosing PCR cycles number and RNA amount in the linear phase of the amplification curve. Bands intensity was analyzed by computerized densitometry. Amplification of GAPDH was used as the internal control in the PCR, and means were compared by Tukey-Kramer-HSD test. FGF-10 expression was detected in all stages of luteal development, but did not differ among them. Mean values (FGF-10/GAPDH ± SEM) were 67.9 ± 17.4 (n=10), 72.4 ± 15.8 (n=10), 52.6 ± 16.4 (n=10) e 95.5 ± 28.3 (n=10) for stages 1, 2, 3 and 4, respectively. In conclusion, present data indicate that FGF-10 expression is not regulated during bovine luteal development. Nevertheless, FGF-10 gene expression in the CL is compatible with a role for FGF-10 in the control of bovine luteal development.

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REGULATION OF FIBROBLAST GROWTH FACTOR RECEPTOR-2b (FGFR-2b) GENE EXPRESSION IN CULTURED BOVINE GRANULOSA CELLS

Buratini Jr., J.[1]; Castilho, A.C.S.[1]; Cao, M.[2]; Nicola, E.S.[2]; Price, C.A.[2]

[1]Department of Physiology, IB, UNESP, Botucatu, Brazil;
[2]Centre de Recherche en Reproduction Animale, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Canada. anthony.castilho@ig.com.br

Fibroblast growth factor receptor 2b (FGFR-2b) is efficiently activated by FGF-7 and 10, which are recognized as paracrine mediators of mesenchymal-epithelial cell interactions that regulate cell proliferation/differentiation. FGF-7 is predominantly expressed by theca cells (TC) and has mitogenic effects on granulosa cells (GC). We recently detected FGF-10 gene expression in oocytes and TC from bovine antral follicles. In the ovary, FGFR-2b is mostly expressed in GC of estrogenic follicles. In the view of the changes in FGFR-2b gene expression during follicle development, it is of interest to identify regulating factors. The objective of this study was to assess effects of FSH and IGF-1 on FGFR-2b gene expression in cultured bovine GC. Small follicles (2-5 mm diameter) were isolated from ovaries obtained from an abattoir, and granulosa cells were placed into serum-free medium supplemented with insulin and bovine FSH (0, 0.1, 1, 10 or 100ng/ml) or IGF-1 analog (0, 5, 10, 50 or 100ng/ml). Cells were cultured for 6 days, with medium changes every 2 days. On Day 6, cells were recovered in Trizol for total RNA extraction. FGFR-2b gene expression was examined by semiquantitative RT-PCR, using abundance of histone 2a (H2a) mRNA as internal control. Experiments were performed on three independent pools of cells and means were compared with the Tukey-Kramer HSD test.. FGFR-2b gene expression (FGFR-2b/H2a mRNA) was higher ($P<0.05$) in cells treated with 10ng/ml FSH (28.1±2.3) compared with 0 or 0.1ng/ml FSH (5.3±1.1 and 4.3±1.1, respectively), and intermediate levels were observed in cells treated with 1 or 100ng/ml FSH (15.8±2.4 and 15.8±4.4, respectively). In IGF-1 treated GC, FGFR-2b gene expression was lower at 10ng/ml (2.4±0.8) in comparison with 0ng/ml (7.2±1.2; $P<0.05$), and intermediate levels were observed in cells treated with 5, 50 or 100ng/ml IGF-1 (5.7±0.6, 4.1±0.5 and 4.4±0.7, respectively). In conclusion, the present data indicate opposite effects of FSH and IGF-1 in the regulation of FGFR-2b expression in cultured bovine GC, and corroborates the hypothesis that this FSH-up-regulated receptor plays an important role during GC differentiation and proliferation in the bovine antral follicle through activation by FGF-7 and FGF-10.

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IDENTIFICATION OF CONEXIN-37 AND CONEXIN-43 IN PREANTRALS FOLLICLES OF OVARIES OF BOVINE FETUSES OF THE NELORE BREED

Basso, A.[1,2]; Tetzner, T.A.D.[2]; Garcia, J.M.[2]; Esper, C.R.[2]

[1] Departamento de Reprodução Animal - FMVZ/USP – São Paulo-SP-Brasil; [2] Departamento de Medicina Veterinária Preventiva e Reprodução Animal - FCAV/UNESP – Jaboticabal-SP-Brasil.
acbusp@hotmail.com

During foliculogenesis, maintenance of junctions between granulosa cells and oocytes is essential for normal development of the follicle (Nuttinck *et al*., Mol. Reprod. Devel. v. 57, p. 60-66, 2000), because they are responsible for transport of supplies and to regulate the entrance of specific proteins (Granot *et al*., Biol. Reprod. v. 66, p. 568-573, 2002). Gap junctions are considered the main responsible for this communication and, in the ovarian follicle, they are directly related to the presence of conexina 37 and conexina 43 since the preantral phase (Simon *et al.*, Nature. v. 385, p. 525-529, 1997). The objective of this study was to identify the presence of Cx37 and Cx43 in junction areas between granulosa cells of bovine fetal preantral follicles in the last gestation quarter. Fragments of fetal ovarian tissue were fastened in Metacarn (60% alcohol metílico + 30% chloroform + 10% glacial acetic acid) for 30 minutes and stored in alcohol 95% for posterior inclusion in paraffin. The primary antibodies were rabbit polyclonal anti conexin37 (anti-Cx37, Alpha Diagnostic) and mouse polyclonal anti conexina43 (anti-Cx43, Zymed Laboratories). The secondary antibody was polyclonal FITC conjugated (Zymed Laboratories). Slides were evaluated by fluorescence microscopy (Nikon E-800, Tokyo, Japan) allowing the detection of Cx37 and Cx43 for the first time in membrane areas corresponding to the junctions between granulosa cells and the oocyte in preantral follicles of bovine fetuses. In conclusion, as well as in adult cows, preantral follicles obtained from bovine fetal ovaries also present specific junctions between granulosa cells and the oocyte, which are composed of conexinas Cx37 and Cx43, providing evidence of the existence of early exchanges between the oocyte and somatic cells during fetal life and indicating metabolic activity in this phase.

EXPRESSION OF THE FULL-LENGTH AND ALTERNATIVELY SPLICED BOVINE LH RECEPTOR mRNAs IN THECA AND GRANULOSA CELLS FROM ANTRAL FOLLICLES

Nogueira, M.F.G.[1*]; Pinto, M.L.G.[2]; Rainho, C.A.[3]; Avellar, M.C.W.[4]; Price, C.A.[5]; Buratini Jr., J.[6]; Barros, C.M.[1]

Departments of [1]Pharmacology, [3]Genetics and [6]Phisiology, IB, and [2]Animal Reproduction, FMVZ, UNESP, Botucatu, São Paulo, Brazil, [5]CRRA, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canadá, [4]Department of Pharmacology, INFAR, UNIFESP-EPM, São Paulo, SP, Brazil. marcelo@femanet.com.br

Growth of dominant follicle in the absence of circulating FSH and the events following the LH surge that culminate in ovulation, are dependent on the interaction between LH and its receptor (LHR). Four LHR alternative transcripts were described in theca and granulosa cells from bovine follicles (Robert C *et al*. 2003 Reproduction **125**, 437-446). Only two of them can be translated to functional proteins (receptors coupled with G protein) with different affinities to their ligands. In humans and marmosets, the full-length isoform has affinity to both LH and hCG molecules, whereas the isoform with deletion of only exon 10 has affinity to hCG exclusively. Additionally, isoforms with deletion of exon 3 were observed in rats, although no previous report have investigated this region of the bovine gene. The objective of this study was to characterize the pattern of gene expression of the LHR in theca and granulosa cells from bovine antral follicles. From ovaries collected in abattoir, antral follicles were dissected (5 to 14mm of diameter), and samples of theca and granulosa cells were obtained to total RNA extraction (Trizol protocol). Steroids concentrations in the follicular fluid were determined by RIA. Gene expression of LHR was measured by semiquantitative RT-PCR with specific primers to amplify part of extracellular region (LHRA; primers annealing on exons 2 and 9) and the fragment from the end of extracellular region, including the transmembrane domain and finishing near the end of intracellular region (LHRBC; primers annealing on exons 9 and 11). As internal control of the PCR, it was used GAPDH expression. Four isoforms were detected from LHRBC fragment: M1 (full-length), M2 (with a deletion of exon 10), M3 (with deletion of part of exon 11), and M4 (with deletions in exon 10 and part of 11). No correlation was observed ($P>0.05$) between LHR isoforms expression in the theca cells and estradiol or progesterone concentration, or follicular diameter. Also, there was no correlation between estradiol concentration and LHR isoforms expression in granulosa cells, however both progesterone concentration and follicular diameter were correlated with LHR expression in these cells. This correlation was observed in the four isoforms amplified from LHRBC fragment (Linear Regression, GraphPad InStat; $r>0.70$ and $P<0.0001$ to progesterone concentration, and $r>0.55$ and $P<0.0004$ to follicular diameter). Thirty seven granulosa cell samples were analyzed, from follicles with diameter of 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14mm (n=3; 4; 6; 6; 4; 6; 4; 1; 2 and 1, respectively). Expression of isoforms from fragment LHRBC was observed in granulosa cells from follicles with 7mm. In 6 follicles with 7mm only one (16.7%) expressed LHR in granulosa cells, whereas most of follicles = 8mm expressed LHR (87.5%, 21/24). No LHR isoforms were detected in granulosa cells from follicles (n=7) with 5 or 6 mm. In the amplicons resulting from LHRA fragment amplification, it was detected the expression of an alternative transcript with deletion of the entire exon 3 (i.e., 75 nt), as previously described in rats. It is concluded that, in granulosa cells from follicles with diameter ≥ 8 mm, the gene expression of the LHR was positively correlated to follicular diameter and progesterone concentration in the follicular fluid. Additionally, sites of alternative splicing were detected in exons 3, 10 and 11 from bovine LHR gene. *Fellowship from FAPESP.

DETECTION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE ENZYME (eNOS) IN BOVINE OVARIES

Natori, M.M.[1]; Santos, N.P.[1]; Adona, P.R.[1,2]; Pires, P.R.L.[1];
Caldas-Bussiere, M.C.[2]; Leal, C.L.V.[1]

[1]Departamento de Ciências Básicas – FZEA / USP – Pirassununga-SP, [2]Laboratório de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, mmnatori@ig.com.br

Nitric oxide is a volatile gas molecule produced by the enzyme nitric oxide synthase (NOS) which is expressed in three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Studies have shown that this gas participates in several cellular functions, among these, some related to reproduction, such as ovulation, oocyte maturation and fertilization (Thaler and Epel, *Curr Pharm Design*, v.9, p.399-409, 2003). The aim of the present work was to determine the presence of the eNOS isoform in bovine ovarian tissue and oocytes before and after *in vitro* maturation (IVM) by immunohistochemistry and immunofluorescence, respectively. For the first procedure (3 replicates), ovaries were fixed in Metacan solution (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin and cut in 5µm sections. Endogenous peroxidase was blocked (3% H_2O_2 in PBS) for 30 min; then unspecific binding was blocked in 20% goat serum in 0.01 M PBS for 1h followed by overnight incubation at 4ºC with the primary antibody (anti-eNOS 1:100 in PBS + 1% BSA). The secondary antibody was added (1:400 in PBS + 1% BSA) for 1 h and then the slides were submitted to the avidin:biotin complex and peroxidase (1:150 in PBS + 1% BSA) for 1h followed by 0.05% DAB + 0.03% H_2O_2 in PBS for 13 min. Nuclei were stained with hematoxylin. For the second procedure (4 replicates), some oocytes were *in vitro* matured for 24h in TCM-199 + 10% FCS, hormones and antibiotics. Another group of oocytes was assessed immediately after aspiration (immature oocytes). Both, immature and mature oocytes were denuded and fixed (3.7% paraformaldehyde and 0.6% Triton X-100 in PBS for 1 h). Then were blocked with 3% goat serum in PBS for 45 min and incubated with the anti-eNOS antibody (1:100) overnight at 4ºC. Next, the oocytes were incubated with the secondary antibody FITC-conjugated (1:100) for 1h followed by staining of nuclei with propidium iodide (10 µg/ml, 15 min). Oocytes were observed under an epifluorescence microscope. As controls in both procedures, the primary antibody was omitted and substituted by PBS only. For immunohistochemistry, six slides were analyzed and in all the oocytes stained positive within primordial to tertiary follicles, as well as vascular endothelium. Three control slides (no primary antibody) were also assessed and no staining was observed. For immunofluorescence, all *in vitro* matured (38) and immature (35) oocytes stained positive. None of the control oocytes (no primary antibody, 19 mature and 13 immature) showed any staining. According to the results, it can be concluded that the bovine ovary expresses eNOS and that the enzyme is present in oocytes throughout folliculogenesis from the primordial follicles to mature oocytes. This is the first report on eNOS detection in the bovine ovary.

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NITRIC OXIDE EFFECT ON GRANULOSA CELLS APOPTOSIS IN ZEBU COWS

Faes, M.R.[1]; Moreira, L.[1]; Carvalho, C.S.P.[1]; Caldas-Bussiere, M.C.[1]

[1] Setor de Reprodução Animal, LMGA, CCTA, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego, 2000, Parque Califórnia, Campos dos Goytacazes, RJ, Brasil, 28013-602, marrfaes@uenf.br

Apoptosis is a process of selective cell deletion implicated as a mechanism underlying the process of follicular atresia. The granulosa cells (GCs) of the follicle are one of the cell types mostly affected by this process. Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS) from L–arginina and O_2 molecular. The role of nitric oxide (NO) has been demonstrated in the ovarian physiology in specially on follicular development, steroidogenesis and follicular atresia. The aim of this study was to verify if NO synthesis by the CGs changes according to the follicle size and time of culture. And to avaluate if a dose - effect–response NO occurs along 24 h, by NO donor (sodium nitroprussid/ SNP), on GCs apoptosis. Ovaries were collected from a local slaughterhouse. Follicles from sizes ranging from small (<5mm), medium (5-8 mm) and large (>8mm) were punctured to obtain antral GCs. CGs were incubated ($5x10^6$) in a well culture 24 plates with 1ml of αMEM/Ham's F12 supplemented with androstenedione (10^{-7}M); penicillin (100U/ml), streptomycin sulfate (100ug/ml), PVA (0.1%), 1% ITS (5ng/ ml de sodium selenite; 5,5ng/ ml de transferrin, insulin 5ng/ ml and 1% of MEM non-essential amino acids 100x solution. FSH was additioned according to the originally follicle size in culture medium at 2 ng/ml (< 5 mm) and 1 ng/ml concentrations (5-8 e > 8 mm). CGs were incubated at 38.5°C for 48 hours (0 h) and 70% of the medium was replacement by medium without (control) or with SNP (10^{-3}, 10^{-5}, 10^{-7} M). After 24 hours, the medium and the CGs were collected to nitrite (NO_2^-) measurement by Greiss reaction and electrophoresis analysis of DNA, respectively. NO_3^- CGs synthesis was not differed ($P>0.05$) among the follicle sizes. However, NO_2^- concentrations increased ($P<0.05$) at 0 to 24 hours at all the groups. All groups with SNP 10^{-3}M presented NO_2^- higher in the culture medium than the groups with others concentrations (10^{-5} e 10^{-7} M) and control group. However, in spite of the high NO_2^- concentration on the culture medium, GCs apoptosis was not induced. Thus, Raised NO concentrations must be used and in an time of bigger culture to verify if NO can be involved in GCs apoptosis of bovine ovarian follicle

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QUANTITATIVE AND MORPHOMETRIC ANALYSIS OF PREANTRAL FOLLICLES OF BUFFALOES FETUSES: PRELIMINARY RESULTS

Santos, S.D.S.[1]; Carvalho, A.C.[1*]; Ferreira, M.A.P.[1]; Silva, M.S.[1]; Silva, E.S.[2*]; Cordeiro, M.S.[2]; Biondi, F.C.[2]; Ohashi, O.M.[2]

[1] Departamento de Histologia e Embriologia - CCB/UFPA, Belém-PA, Brasil
[2] Laboratório de Fertilização *in vitro* - CCB/UFPA, Belém-PA, Brasil
* Bolsista PIBIC - UFPA / PIBIC - CNPq damasc@ufpa.br

Preantral folicllles are a major source of oocytes, and their utilization as an important tool to store large number of female gametes for future use in reproductive programs has been investigated. The folliculogenesis in buffaloes is poorly understood, there are only few works about its beginning during the fetal stage and follicle population in adults females, with no one report about quantification and morphometry during prenatal phasis. The present study aims to determine the quantification and morphometry of preantral follicles of buffaloes fetuses. Eight buffalo ovaries from fetuses of 5 and 6 months of age, determined according ABDEL-RAOUF & EL-NAGGAR (**UAR J. Vet. Sci., 5**, 37-43, 1968), were collected at slaughterhouse and processed for classical histology. To follicle quantification were used the methodology described by GOUGEON & CHAINY (**J. Reprod. Fertil., 81,** 433-442, 1987) and to determine the oocyte and follicle diameters was used a micrometric ocular Zeiss with a optic microscope (OLYMPUS CHC20). The preliminar data analysis showed a population of the 48,612.42 (±17,301.96), 25,071.25 (±10,578.32) and 724.85 (±375.54) primordial, primary and secondary follicles per ovary, respectively. The follicular, oocyte diameters and the number of follicular cells were, respectively, of 28.9 ±3.4 µm , 21.66 ±2.81µm and 7,12 ±1,4 (primordial follicles); 34.68 ±5.89 µm, 24.3 ±3.43µm and 11.97 ±2.44 (primaryl follicles) and 59.38 ±12.6µm, 33.0 ±7.66µm and 13.76 ±2.43 (secondary folliclesThe present study described the quantification and morphometry of preantral follicles of buffalo fetuses, showing that the folliculogenesis has been established with the presence of the primordial, primary and secondary follicles at the 5 th and 6th month of age.

CRYOPRESERVATION OF BOVINE PREANTRAL FOLLICLES

Celestino, J.J.H.[1]; Santos, R.R.[1]; Matos, M.H.T.[1]; Martins, F.S.[1]; Costa, S.H.F.[1]; Silva, J.R.V.[1]; Rodrigues, A.P.R.[1]; Figueiredo, J.R.[1]

[1] Laboratory of Manipulation of Oocytes Enclosed in Peantral Follicles – LAMOFOPA – FAVET - UECE, Fortaleza-CE, 60740-000, Brazil. htmatos@yahoo.com

This study evaluated the viability of bovine preantral follicles (PF) cryopreserved with or without previous conservation of the ovarian tissue in 0.9% saline solution at 4 °C for 24 h. Each bovine ovarian pair was divided in 39 fragments. One fragment was immediately fixed and the remaining fragments transported at 4 or 20 °C in 0.9% saline solution. At laboratoty, one fragment transported at 20 °C was immediately destined to follicular isolation and analysed with Trypan Blue, and the remaining fragments transported at the same temperature were submitted to toxicity test (TT) or cryopreservation in Glycerol (GLY), Propanediol (PROH), Ethyleneglycol (EG) or DMSO at 0, 1.5 or 3.0 M. The fragments transported at 4 °C were kept in this temperature during 24 h and submitted to the same procedure developed to that ones transported at 20 °C. At the end of each treatment, the fragments were destined to follicular isolation, and the suspensions evaluated with Trypan Blue. PF were classified as viable (not stained) or not viable (stained). The percentages of viable PF were compared using Chi-square at 5% of probability. It was not observed significant reduction on the percentage of viable PF after transport at 4 °C (80%) as well as 20 °C (78%) when compared to the control (85%). Similar results were observed when PF were exposed at the medium without cryoprotectant or in EG 1.5 M without previous conservation at 4 °C. After cryopreservation, the higher rates of viable PFs were obtained when the ovarian fragments were frozen in presence of EG 1.5 M with (77%) or without (74%) previous cooling at 4 °C, as well as in DMSO 1.5 M without previous cooling for 24 h (76%). In conclusion, previous cooling procedure doesn't affect negatively the cryopreservation, since the bovine ovarian fragments are frozen in presence of EG 1.5 M.

VIABILITY OF PRIMORDIAL FOLLICLES FROM CRYOPRESERVED OVARIAN CORTICAL TISSUE IN SHEEP.

Tsuribe, P. M.; Landim-Alvarenga, F.C.

1 Departamento de Reprodução Animal e Radiologia Veterinária FMVZ/UNESP, Botucatu-SP, 18618-000, Brasil. pmtsuribe@ig.com.br

Recent improvements in the treatment of cancer by chemotherapy and radiotherapy have led to a significant increase in the survival of young cancer patients. However, these treatments have a highly deleterious effect on the ovary causing a severe depletion of the follicular store. Freeze fragments of ovarian cortex prior to treatment could be an alternative to preserve fecundity. The cryopreservation of ovarian tissue could be applied in transplantation of ovarian cortex to restore cyclic production of oocytes or, through culture and *in vitro* maturation of frozen primordial follicles to obtain mature oocytes for *in vitro* fertilization. Just a few studies compare the utilization of different cryoprotectors (DMSO, EG, PROH and GLI) and theirs toxic effects on ovarian tissue. The main objective of this experiment was to compare the effect of the cryoprotector DMSO, EG and their association on sheep pre-antral follicles before and after freezing. Fifty (50) ovaries from ewes with 12 to 24 months of age were utilized. Follicular viability was analyzed though the use of Hoescht 33342 and propidium iodite, just after collection, after toxicity test and cryopreservation as well as at the end of culture. After the isolation the number of viable primordial follicles was 78.9% in the control group. The percentage of viable follicles in the toxicity test using EG (1.5M), DMSO (1.5M) and EG+DMSO (1.5M) was 77.1%, 68.4% and 60,7% respectively. After cryopreservation, the viability was reduced to 75% for EG, 60% for DMSO and 55.6% for the association. The average number of isolated primordial follicles after cryopreservation in all treatments (13.3 ± 5 for EG; 10.1 ± 6 for DMSO and 5.9 ± 4 for EG/DMSO) was lower than fresh tissue (23.2 ±8). In the first day of culture, it was observed that the follicles changed their aspect but remain translucent. The granulosa cells originated from follicles surface adhered to the dish and proliferated, forming a monolayer around the follicles. On the 10º day of culture, the initial follicular structure was lost and the follicles acquired a bidimentional growth. On day 0, morphologic differences were not observed between follicles isolated from fresh, submitted the action of cryoprotectors and freeze thawed with EG and DMSO tissue. Nevertheless, the follicles frozen with EG+DMSO showed signals of degeneration. For fresh follicles, as well as those submitted the action of EG, the follicular viability in the 1º day of culture was similar (66.4% for control and 62% for EG), but lower than day 0 (78.9%). However, after 6 and 10 days there was a reduction in the percentage of follicles considered viable in all groups. In the case of follicles submitted the action of cryoprotectors DMSO and EG+DMSO, as well as the frozen samples, the follicular viability was significantly reduced compared to the control group. In the conditions of this experiment *in vitro* follicular growth was observed in all groups. However the in vitro culture damaged follicles in all groups. The treatment with EG offer similar results compared to the control group.

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ULTRA STRUCTURAL CHARACTERIZATION OF BUFFALO (*Bubalus bubalis*) PREANTRAL FOLLICLES

Mondadori, R.G.[1,2]; Luque, M.C.A.[1]; Santin, T.R.[2]; Báo, S.N.[1]

[1]Department of Cellular Biology, Institute of Biological Science, University of Brasília, Brasília, DF 70910-900, Brazil; [2]Animal Reproduction Laboratory, Veterinary Medicine Departament, UPIS – Faculdades Integradas, SEPS EQ 712/912, Conjunto A, Brasília – DF, 70390-125.
mondadori@upis.br

The mainly objective of the present experiment was to characterize the buffalo preantral follicles. This characterization is important to provide more knowledge in the reproductive technology used to optimize the use of buffalo female genetic. Parts of ovarian cortex, collected from cycling buffalo females, were selected under stereomicroscopy and processed for optic and transmission electron microscopy. The ovarian tissue blocks were fixed in a 0.1M sodium cacodilate buffer solution, containing 2% of glutaraldehyde and 2% of paraformaldehyde at 4ºC during 24 hours. The post-fixation was performed with osmium tetroxide and contrasted *in block* with uranile acetate. After this, the tissue was dehydrated in an acetone series. In order to elaborate the semi-thin sections (3μm), the tissue blocks were included in Spurr. To classify the preantral follicles and determine the nuclear region, the sections were dyed with toluidine blue and observed under light microscopy. The ultrathin sections (70nm) were made from follicles with intact nucleus and contrasted with uranil acetate and lead citrate to be observed in transmission electron microscopy (Jeol 1011). Primordial follicles were characterized as an oocyte surrounded by one layer of flattened cells. The buffalo primordial follicle has a mean diameter of 35.02 μm and the oocyte diameter is 24.87 μm. The oocyte nucleus is relatively large and eccentric; in the cytoplasm, a large amount of mitochondria, vesicles and endoplasmic reticulum cistern, mainly smooth is observed. The primordial follicles cells are rich in plasmatic membrane invaginations, these invaginations are observed within the cell and between the cell and the oocyte. At this follicular stage the begging of zona pellucida deposition can also be seeing in areas between the oocyte and follicular cells. The primary follicles (mean diameter of 41.80 μm) consist in an oocyte, with a medium diameter of 26.91 μm, surrounded by one layer of cubical cells. In these structures a more important amount of zona pellucida glycoprotein can be observed, and also appeared some gap junctions between the oocyte and granulosa cells. The secondary follicles, which are surrounded by more than one layer of cubical cells, have a diameter of 53.35 μm, and the oocyte has a medium diameter of 29.38 μm. The ultra structural analysis showed a large amount of coalescent vesicles, more evident in the oocyte periphery. The zona pellucida is thicker at this stage and with a large quantity of glycoproteins. This analysis showed that the ultra structure of buffalo and bovine preantral follicles are similar, except that in buffalo there is a larger amount of cytoplasmic vesicles since the beginning of oocyte development. Besides, buffalo oocytes and preantral follicles have a smaller diameter when compared to bovine, mainly in primary and secondary follicles (Meirelles, *et al.* **Anim. Rep. Sci. 87:** 45-57, 2005).

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ULTRASTRUCTURAL ANALYSIS OF OVINE PREANTRAL FOLLICLES CULTURED IN THE PRESENCE OF INDOL ACETIC ACID, EGF AND FSH

Andrade, E.R.[1]; Landim-Alvarenga, F.C.[2]; Blaschi, W.[3]; Alfieri, A.A.[3]; Seneda, M.M.[3]; Figueiredo, J.R.[1]; Toniolli, R.[1]

[1] Faculty of Veterinary Medicine, PPGCV, State University of Ceara, Fortaleza-CE, 60740-000, Brazil. [2] São Paulo State University - UNESP, FMVZ, Botucatu-SP, 18618-000, Brasil. [3] State University of Londrina, Londrina-PR, 86051-990, Brazil. wblaschi@hotmail.com

The objective of this experiment was to determine the ultrastructural features of ovine preantral follicles cultured in the presence of indol acetic acid (IAA), epidermal growth factor (EGF) and follicle stimulating hormone (FSH). The ovarian cortex was divided into small fragments that were cultured for 6 d in culture plates with: Minimum Essential Medium (MEM) supplemented with ITS (insulin-transferrin-selenium), pyruvate, glutamine, hypoxantine, bovine serum albumine and antibiotics (MEM+); MEM+ plus IAA (40 ng/mL); MEM+ plus IAA+EGF (100 ng/mL) or MEM+ plus EGF+FSH (100 ng/mL). After 6 d of culture in each treatment, ultrastructural analysis was performed on preantral follicles from all groups. Only follicles classified as morphologically normal in the semi-thin sections were evaluated. Briefly, small pieces of ovarian cortex were fixed in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in the same buffer. Subsequently, samples were dehydrated in increasing series of acetone and embedded in Epon resin. Semi-thin sections (3 mm) were stained with toluidine blue. Thin sections (80 nm) were obtained with a diamond razor, mounted in copper grids, contrasted with uranyl acetate and lead citrate and examined using a Philips CM100 transmission electron microscope. Preantral follicles cultured in MEM with IAA+EGF or EGF+FSH exhibited a healthy oocyte surrounded by one or more well-organized layers of granulosa cells. The ooplasm contained numerous rounded or elongated mitochondria showing few mitochondrial cristae. Alter shape mitochondria were also seen, indicating the multiplication of this organelle. Golgi complexes were rare, but well-developed. Both smooth and rough endoplasmic reticulum was observed, either as isolated aggregations or forming associations with mitochondria and vesicles. A variable number of vesicles spread throughout the ooplasm were also observed. Sometimes microvilli were observed in the oolema and, occasionally, small amounts of zona pellucida material were visible. At all developmental stages a thick layer of glycoproteins, the basement membrane, surrounded the preantral follicles. Granulosa cells were small, with high nuclear:cytoplasmic ratio. These cells showed many elongated mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus, some electron dense vesicles were also seen. In conclusion, ovine preantral follicles can sustain their health and viability for up 6 d when cultured in media supplemented with IAA and EGF or EGF and FSH.

THE EFFECT OF DIFFERENT CONCENTRATIONS OF 3 INDOL ACETIC ACID IN THE BOVINE PREANTRAL FOLLICLES CULTURE: PRELIMARY RESULTS

Blaschi, W.[1]; Andrade, E.R.[2]; Delboni, M.I.[1]; Boselli, C.C.[1]; Bracarense, A.P.F.R.L.[1]; Alfieri, A.A.[1]; Figueiredo, J.R.[2]; Seneda, M.M.[1]

[1] Universidade Estadual de Londrina, Londrina-PR, 86051-990, Brasil.
[2] Faculdade de Medicina Veterinária, PPGCV, Universidade Estadual do Ceará, Fortaleza-CE, 60740-000, Brasil. wblaschi@hotmail.com

This experiment was carried out in order to value the activation and growth of bovine preantral follicles after we have done *in vitro* culture of ovarian cortex in different concentrations of indol acetic acid (IAA). In a slaughter-house, 15 minutes far from the laboratory, we obtained a Nelore calf ovary pair. The ovaries were washed in alcohol 70%, 0.9% saline solution and immediately transported to the laboratory in physiological solution supplemented with penicillin (200 UI/mL) and streptomycin (200 mg/mL) at 39 °C. The ovarian cortex was divided into fragments with 5mm x 5mm approximately (1mm thickness). One fragment was fixed in Carnoy (control-time zero – D0) and the others were cultivated for 2 or 6 days in Minimum Essential Medium (MEM) supplemented with ITS (insulin – transferrin – selenium), piruvate, glutamine, hypoxantine, bovine serum albumine and antibiotics (MEM+) or they were cultived in MEM+ plus 10, 40, 100, 500 or 1000 ng/mL of IAA. After culture, the ovarian fragments were fixed in Carnoy, dehydrated in ethanol, clarified with xylol and incubated in paraffin. For each cortex fragment we did slats with tecidual sessions of 5µm and we used hematoxylin-eosyn method to bleach these slats. Follicles were classified in agreement with growth phase as primordial or growing follicles (primaries and secondaries). In each treatment, after 2 or 6 days of culture we analyzed 30 follicles. The results showed a reduction in the proportion of primordial follicle (25% - 3/12) associated with an increase of growing follicles (75% - 8/12) for the treatment supplemented with 40 ng/mL of IAA after two days which indicate activation process. Similar results were observed for the MEM+ plus 10 ng/mL of IAA during 6 days of culture treatment. However, the treatment supplemented with 1000 ng/mL of IAA after 2 or 6 days of culture showed respectively that 80% (24/30) and 86.7% (26/30) of preantral follicles presented some degeneration. The control treatment (D0) showed 30% (9/30) normal morphologically preantral follicle of which 55.5% (5/9) of these follicles were classified as primordial and 44.5 % (4/9) were classified, respectively, primordial and growing. We observed that bovine primordial follicles can be activated in vitro after the culture in MEM+ plus 40 ng/mL of IAA after 2 days or culture with 10 ng/mL of IAA after 6 days and the high IAA concentrations can be damaging to this follicles. These results are preliminary and a better analysis can be possible with the continuity of the experiment.

EMBRYO CULTURE CONTAMINATION USING COMMERCIAL SEMEN SAMPLES: CASE REPORT

D'Angelo, M.; Vieira, S.E.M; Pavão, D.L.; Malaguti, J.M.A.; Rojas, N.; Souza, R.J.; Athayde, C.; Ruyz, D.C.; Galuppo, A.G.; Pinheiro, E.S.

Centro de Sanidade Animal-Instituto Biológico de São Paulo, São Paulo-SP, 04014-002, Brasil
dangelo@biologico.sp.gov.br

Semen is a potential source of non-pathogenic, as well as pathogenic microorganisms. Contamination in the semen collection procedure is relatively low , but is not necessarily germ-free requiring the use of antibiotics . However, it is important to understand that some microorganisms may be resistant and antibiotics will not be effective. Units of semen containing *Stenotrophomonas maltophilia, Pseudomonas putida, Pseudomonas aeroginosa, Enterobacter cloacae, Staphylococcus sciuri, Acinectobacter cacloaceticus, Pantoeau agglomerans ou Flavobacterium spp* have been reported to contaminate IVF embryos cultures (Stringfellow, Theriogenology, 53: 85, 2000). The aim of this work was report that the acceptable microorganisms concentration in a semen sample for insemination could not be safe for an *in vitro* fertilization (IVF). The IVF procedures were performed with oocytes collected from slaughterhouses bovine ovaries, *in vitro* matured. Semen samples were thawed and processed in Percoll (CULTILAB-Campinas, SP). Oocytes were washed in HTF + 10%SFB and kept with the spermatozoa for fertilization. After fertilization, it was verified the presence of contamination, in two consecutive IVF procedures, performed with two different semen samples that were apparently normal. To locate the main focus of that contamination, a third IVF was performed, with the same samples used before. The IVF procedure was performed according the normal protocol. Fertilization dishes were prepared with four drops each. In the drop 1 it was done a conventional IVF, in drop 2 it was kept just oocytes, in drop 3 just semen and the drop 4 was used for medium control. The dishes were maintained in CO_2 incubator for 18h. The presence of contamination was confirmed in drops 1 and 3 of each dish, showing that probably the semen was the contamination focus. Culture tests were performed in those two semen samples, with other 7 semen samples from other batch that have not been used yet. The oocytes and a medium sample were also tested in culture, and presented negative results. Results of the two first semen samples were positive, with growing of: *Acinetobacter spp (5000 ufc), Enterobacter aerogenes (5000 ufc), Escherichia coli (5000 ufc) and Streptococcus spp (uncountable ufc).* To the other 7 semen samples it was found 3 negative and 4 positive results, with growing of: *Acinetobacter spp (300ufc e 1600 ufc), Enterobacter aerogenes (uncountable ufc) and Alcaligenes faecalis (uncountable ufc).* The presence of those bacteria in the semen samples make the IVF procedure not viable. Each new development of animal reproduction techniques new challenges on the disease control appears. Therefore, it is important to have in mind the quality control patterns established for old techniques should not be consider safe for the new ones, as in the case of microorganisms concentration in semen samples, which could be used for insemination or IVF. Apparently for IVF the semen sample should be completely germ-free, because the in vitro condition of the system promote an excellent ambient for it proliferation.

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FIRST RESULTS WITH IVF USING SEXED SPERMATOZOA IN BRAZIL

Oliveira, K.M.[1,3]; Franco, R.V.R.[1]; Diniz, E.G.[3]; Vieira, F.V.[2]; Galassi, A.G.[1]; Tavares, M.[4]; Braga, M.Q.[1]; Galeli, G.[1]; Rodrigues, L.F.[1]

[1] Goyaike Brasil Agropecuária Ltda – Sexed Semen Laboratory – Uberaba – MG – Brazil. [2] ABS PecPlan – Sêmen Laboratory – Uberaba - MG-Brazil. [3] Animal Reproduction Department – FAMEV/ UFU, Uberlândia – MG, Brasil. [4] Mats Department - FAMAT/ UFU, Uberlândia – MG, Brazil
kmello@goyaike.com / rfranco@goyaike.com

The *in vitro* fertilization (IVF) or *in vitro* embryos production in bovines is one technique that includes the process of maturation, fecundation and embryonic development allowing the improvement of other techniques of the animal reproduction like cloning and gene transference. The technology is used to accelerate the production of high genetic merit animals and also allowing the use of females with acquired alteration and permitting deepening the knowledge of biotechnological processes. One of the challenges of the biotechnology of the animal reproduction and its success that has been searched intensely since the discovery of artificial insemination was the sperm sexing technology providing great benefits for the genetic improvement. The sperm sexing is based on the flow cytometric separation of X and Y chromosome bearing sperm based on X/Y DNA content difference. In cattle the X bearing sperm contains 3.8 percent more DNA than Y bearing sperm. Therefore the application of the sperm sexing in cattle constitutes an enormous advance in animal reproduction. The sperm sexing for flow cytometric is a tool that can be applied with IVF and other techniques allowing eliminate one limitation found in the IVF: a bigger number of births of males in relation to females. The objective of this work was to tell the results about *in vitro* fertilization using sex specific semen (sexed semen) by different laboratories with Nelore and Brahman bulls in Brazil. The method used for preparation of the spermatozoa for IVF was the Percoll gradient. Staws of sexed sperm of three bulls Nelore and one Brahman have been used in IVF. The *in vitro* fertilization was done by four different laboratories. A total of 2717 viable oocytes had been inseminated with respective semen producing 50.2% and 23.6% of cleavage rate and blastocyst rate, respectively. The data were analyzed by test t student ($p \leq 0.05$). The results show the viability of using the sex sorted sperm in IVF presenting similar results with the unsorted sperm.

IN VITRO PRODUCTION (IVP) OF BOVINE EMBRYOS USING SEXED SPERMATOZOA

Brum, D.S.[1]; Leivas, F.G.[1]; Saliba, W.P.[2]; Alvim, M.T.T.[2]

[1] Nova Índia – Cenatte Embriões, Uberaba-MG. [2] Cenatte Embriões, Pedro Leopoldo-MG
danisbrum@yahoo.com.br

Sexed spermatozoa commercialized in Brazil since August (2004) is an important tool for production of females. However, prudence is necessary for its introduction in business applications regarding costs and variable results reported in other countries. Until the moment, our laboratory has made use of semen from five bulls of different breeds for IVP of embryos: Holstein (HO), Pooled Nelore (NM), Brahman (BR), Nelore (NE) and Girolando (GO). After selection, 259 oocytes obtained by ovum pick-up (OPU) from 10 donors (HO, NM, BR, NE and GO) were matured in modified TCM 199 for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air. In vitro fertilization (IVF) was performed for 18-22 hours with FERT-TALP supplemented with PHE and heparin. Semen was thawed and processed over a Percoll gradient and insemination performed with $2x10^6$ sperm/mL in the same atmospheric conditions of IVM. *In vitro* culture (IVC) was performed in SOF medium supplemented with protein source for 6 days at 39°C in a satured humidified atmosphere of 5% CO_2 in air. Cleavage and blastocyst formation rates were determined on day 2 and 7 after the onset of in vitro culture, respectively. Pregnancies and sex determination were determined at 60 days. Procedures performed with semen from bull GO showed contamination after IVF and was discarded in this work. Cleavage formation rates were 100% (14/14), 100% (66/66), 77% (46/60) and 50% (43/86) for breeds HO, NM, BR and NE, respectively. Percentage of transferable embryos per number of oocytes were 50, 79, 40 and 22% and pregnancy rates were 57, 46, 33 and 53% for HO, NM, BR and NE bulls, respectively, resulting in 83% (38/46) being determined as females. Median embryo production results (45%; 102/226) and pregnancy rate (45%; 46/102) obtained until the moment is similar to those reached with non-sexed semen in our laboratory. Utilization of these bulls semen in a higher amount of procedures, as well as different batch, may certify the reproducibility of these results.

IN VITRO PRODUCTION OF BOVINE (*Bos taurus indicus*) EMBRYOS USING SEXED SPERMATOZOA

Ereno, A.F.C.; Andrade, R.F.; Pontes, J.H.F.

In Vitro Brasil, Mogi Mirim-SP, 13800-970, Brasil. josehenrique@invitrobrasil.com.br

The objective of this study was to evaluate the use of sexed spermatozoa (flow cytometry/cell sorting) in *in vitro* production of bovine embryos in different zebu breeders. The oocyte was aspirated from zebu donor cows (Nelore, Brahman and Gir). They were maturated for 24 h (38,5° C and 5% CO_2) in TMC 199 supplemented with hormone and 10% of fetal calf serum. Eighteen hours post in vitro insemination fertilized oocytes were cultured in SOF medium at 38,5°C in 5% CO_2. Sexed semen were thawed at 35° C in water and separated at Percoll gradient (45 and 90%). It was evaluated the cleavage, embryo production, percentage of pregnant and fetal sexing rates. The cleavage rate and embryo production were 52% and 31%, 56% and 34% and 55% and 34% for Nelore, Brahman and Gir respectively. The percentage of pregnant and female fetal sexing rate were (32% and 95%), (36% and 96%) and (34 and 96%) for Nelore, Brahman and Gir respectively. In conclusion, the sexed semen technology in commercial IVF programs provided satisfactory results and this technology can contribute to increase the number of females in the progeny.

IN VITRO PRODUCTION OF BOVINE EMBRYOS USING SEXED SPERM

Tonello, T.T.M.[1]; Accorsi, M.F.[1]; Ferraz, M.L.[1]; Watanabe, M.R.[1]; Meirelles, F.D.P.[2]; Meirelles, F.V.[2]; Watanabe, Y.F.[1,2]

[1]Vitrogen® Pesquisa e Desenvolvimento em Biotecnologia da Reprodução, Cravinhos–SP, Brasil.
[2]Depto de Ciências Básicas da FZEA-USP, Pirassununga-SP, Brasil.
tatiana@vitrogen.com.br

The continuous growth of Brazilian cattle breeding has been generating great expectations regarding new biotechnologies that are being placed in the market. The use of sexed sperm added to other biotechniques such as IVF is, therefore, a very important tool to intensify and increase the genetic improvement of animals of high genetic value. The aim of the present work was to assess the efficiency of sexed sperm used in the process of *in vitro* fertilization. Thirty doses of sexed semen from nine different bulls were analyzed, mated with 168 donors to fertilize 3,622 oocytes. The oocytes were matured in TCM 199 medium added with FSH, LH, Estradiol and 10% FCS for 24 h at 38.8°C and 5% CO_2 in air. Semen was thawed in water-bath at 35°C and sperm placed in a tube containing the Percoll gradient (45% and 90%) to separate motile cells. Sperm was centrifuged at 250 g for 30 min and, next, viability and motility of spermatozoa were assessed. The inseminating dose was $1x10^6$ sperm cells/ml and co-culture (spermatozoa and oocytes) was in droplets of IVF medium (TALP + PHE + heparin 10 µg/ml) at 38.8°C and 5% CO_2 in air for 18 to 22 h. Presumptive zygotes were cultured in CR2 medium with 10% FCS under the same temperature and gas conditions. Data were analyzed by ANOVA and means compared by TUKEY-KRAMER HSD test. Cleavage rate assessed on the third day presented a mean of 53.2% and a highly significant difference among bulls ($P < 0.0001$) with a variation of 6.2 ± 20.1% to 70.6 ± 29.5%. The mean blastocyst rate assessed on days 7 and 8 of culture was 13.8% and also presented a significant difference ($P<0.01$), with a variation among bulls of 0.0 ± 8.4% to 52.9 ± 14.6%. However, pregnancy rate was not significantly different for the three bulls that presented pregnancies ($P>0.05$), despite the variation of 0 to 52 ± 0.1%, with a mean of 40.7%. It can be concluded that there is a great variation in cleavage and blastocyst rates when sexed sperm is used. However, the great variation between and within lots should be mentioned, indicating that studies must be developed to improve protocols and viability of sexed sperm.

EFFECT OF SPERMATOZOA ON THE *IN VITRO* MATURATION FROM BITCHES (*Canis familliaris)* OOCYTES

Rocha, A.A.[1]; Bastos, R.[1]; Cunha, I.C.N.[1]; Quirino, C.R.[1]; de Souza, D.B.[2]; Paes de Carvalho, C.S.[1]

[1] Laboratório de Melhoramento Genético Animal – Universidade Estadual do Norte Fluminense Av. Alberto Lamego, 2000 – Horto - Campos dos Goytacazes – RJ- Brasil amanda@uenf.br, rocha_aa@yahoo.com.br; [2] Clínica Cirúrgica e Cirurgia - Hospital Veterinário - Universidade Estadual do Norte Fluminense

In the use of reproductive biotechnologies to the canids, the recovered oocytes of ovarian follicles of the females represent an important source of studies that involve the development of female gametes. The *in vitro* maturation (IVM) of bitch oocytes has lower taxes of success when compared with other mammals, being that the presence of spermatozoa can influence in the process of IVM. The aim of this study was to optimize the IVM of bitch oocytes, evaluating the effect of the co-incubation between spermatozoa and oocyte in the retaken of oocyte meiosis. For this study were used 47 bitches from the routine of the Veterinary Hospital of the State University of North Fluminense (UENF) and of the Animal Disease Center Control from Campos dos Goytacazes – Rio de Janeiro. The ovaries had been taken to the Laboratory of Animal Genetic Improvement (LMGA/UENF) and the oocytes gotten from slicing de ovarian tissue. The oocytes were classified in degree I, degree II and degenerate in accordance with the quality (HEWITT et al., *Theriogenology.* 49: 957-966, 1998), after this the oocytes of degree I had been selected for the process of IVM. The medium used from IVM was prepared daily with TCM 199 with 10% serum of estrous bitch and antibiotics. The experiment was divided In two groups: treat group, being the immature oocytes incubated for 24 hours with canine spermatozoa and after this period the oocytes had been submitted the IVM for 48 hours, completing 72 hours of culture. And the control group that the oocytes has been submitted to the IVM for 72 hours without contact with canine spermatozoa. The data had been compared by the ANOVA, followed of test from medium comparison. The control and treat groups with canine spermatozoa during the process of the IVM showed 4.91% and 19.96% of the maturation, respectively, being that the maturation in treat group was significantly increased ($p<0.05$) compared to control group. During the process of co-incubation between spermatozoa and oocytes, the pellucid zone of oocytes is a place of great importance for the interaction between the masculine and feminine gametes. In accordance with EPPIG (EPPIG, J.J. *Reproduction*, 122:829-838, 1991) the strong union of the cells of *cumulus* of the oocytes of mouse realized inhibitory effect in process of the maturation being reverted at the moment that it initiates the expansion of the layer of cells of *cumulus*. HAY (HAY M.A., Doctoral thesis - The Faculty of Graduate Studies of The University of Guelph - Toronto, 182p, 1996 describes some authors that have discussed the interaction between spermatozoa and the pellucid zone of oocytes stimulating the synthesis of the maturation promote factor (MPF) in the oocyte cytoplasm in bovine, suine and ovine. The MPF synthesized in ooplasm induces the germinative vesicle breakdown, retaking the meiotic division until the MII. The results allow to conclude that the presence of spermatozoa during the process of IVM can induce the retaking the meiotic suggesting that the interaction stimulated for the dog spermatozoa can control cytoplasmic factors of feminine gamete.

SPERM PENETRATION IN PIG OOCYTES SUBMITTED TO DIFFERENT MATURATION SYSTEMS

Lima, A.S.; Nascimento, A.B.; Oliveira, V.P.; Marques, M.G.;
Assumpção, M.E.O.A.; Visintin, J. A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
aballarotti@yahoo.com.br

This study was conducted to evaluate sperm penetration, polispermy and pronuclear formation in pig oocytes submitted to three different systems of *in vitro* maturation (IVM). Follicles from ovaries were aspirated and the oocytes subdivided in three groups with 44 hours of IVM, using NCSU23, Whitten and TCM199 media. After the IVM, the oocytes were fertilized in MTBM for six hours, cultivated in NCSU23 for 18 hours, settled in paraformaldehyde 4% and stained with Hoechst 33342 to evaluate polispermy. Sperm penetration rates were 85.8% in NCSU23, 79.7% in Whitten and 80.3% in TCM199, not differing significantly ($p<0.05$). The polispermy rates had no difference among groups ($p<0.05$), which were of 78.4%, 68.6% and 70.6% for NCSU23, Whitten and TCM199, respectively. The pronuclear formation was 78.3% in NCSU23, 75.4% in Whitten and 100% in TCM199, not showing difference ($p<0.05$). In conclusion, oocytes penetrability, high polispermy rates and pronuclear formation had not been affected by treatments of *in vitro* maturation, but new approaches are needed to be adopted to minimize the polispermy in pig embryos.

INFLUENCE OF DIFFERENT SPERM CONCENTRATIONS AND PROTEIN SOURCES ON CLEAVAGE AND IN VITRO EMBRYOS DEVELOPMENT RATES IN PIGS

Nascimento, A.B.; Marques, M.G.; Oliveira, V.P.; Coutinho, A.R.S.; Gonçalves, J.S.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
aballarotti@yahoo.com.br

The aim of this work was to evaluate cleavage and development rates of in vitro fertilized embryos by different sperm concentrations and cultivated in medium with fetal calf serum (FCS) or bovine serum albumin (BSA). Concentrations of $2x10^3$ or $4x10^3$ sptz/oocyte for IVF and NCSU23 medium supplemented with 3 mg of BSA/mL for embryo culture until day 5 after fertilization (D5) were used. On D5 the embryos was divided in two groups: NCSU23 with 10%FCS and NCSU23 with 3mgBSA/mL. Cleavage on D3 and blastocyst from D6 to D8 of culture were evaluated. For statistical analyze was used ANOVA and Tukey test ($P<0.05$). No differences were observed in cleavage rates on D3 between $2x10^3$ (56.8%) and $4x10^3$ sptz/oocyte (56.9%). Difference was observed in blastocyst rates between NCSU23 with FCS (12.8 and 11.8%) and NCSU23 with BSA (17.1 and 16%), respectively, for $2x10^3$ and $4x10^3$ sptz/oocyte. In conclusion, different sperm concentrations can be used for *in vitro* fertilization and NCSU23 with BSA can improve *in vitro* pig embryos production.

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EFFECT OF BOARS, EJACULATES, EXTENDERS AND CAFFEINE ON THE RATES OF IN VITRO SPERM CAPACITATION

Nascimento, A.B.; Nichi, M.; Coutinho, A.R.S.; Marques, M.G.; Oliveira, V.P.; Gonçalves, J.S.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil aballarotti@yahoo.com.br

Boars are recognized as a significant source of variation for success of in vitro embryos production in pigs. As a result, numerous studies have investigated relationships among boars and ejaculates for the establishment of sperm capacitation protocol that results in high rates of monospermic embryos and with adequate development. The search of this protocol has found difficulty, because of the great individual variability among ejaculates and boars. The present experiment was designed to evaluate sperm capcitation among three boars A, B and C and among six ejaculates of each animal. Two extenders were used, Beltsville Thawing Solution (BTS) and Dehydrated Coconut Water (ACP250®), associated (CAF) or not (CTL) with caffeine, resulting in four groups: T1 (ACP250®+CTL), T2 (ACP250®+CAF), T3 (BTS+CTL) and T4 (BTS+CAF). For the statistical analysis it was used ANOVA and the Tukey test with significance level of 5%. Difference was detected among groups without caffeine (T1=18% and T3=18.5%) in relation to the groups with caffeine (T2=44.8% and T4=45.9%), independently of the extender ($p<0.05$). However, no difference was observed among boars (A=38.6%, B=34.6% and C=22.2%) and among ejaculates of each boar ($p>0.05$). In conclusion, there was no influence of boars or ejaculates, but there was influence of caffeine on sperm capacitation rates.

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EFFECTS OF ANTIOXIDANTS DURING *IN VITRO* FERTILIZATION ON QUALITY AND FERTILITY OF BOVINE SPERMATOZOA

Gonçalves, F.S.[1]; Barretto, L.S.S.[1]; Arruda, R.P.[2]; Mingoti, G.Z.[3]

[1] Faculty of Agrarian and Veterinary Sciences, UNESP, Jaboticabal-SP, Brazil. [2] FMVZ, USP, São Paulo-SP, Brazil. [3]Veterinary Medicine, UNESP, Araçatuba-SP, 16050-680, Brazil.
viuva_vet98@yahoo.com.br

Free oxygen radicals (ROS) generated *in vitro* react with cell proteins, lipids and DNA resulting in cell alterations, which can also damage the spermatozoa. It has been demonstrated that ROS can lower sperm motility, but on the other hand, it has been shown that ROS can be involved in the process of sperm capacitation. Thus, we decide to investigate the effect of the antioxidants 2-Mercaptoetanol (2-ME) and Cysteamine (Cist) during *in vitro* fertilization (IVF) on sperm morphology and fertilizing capacity, assessed by evaluation of embryo cleavage after IVF. Oocytes (n=230) were matured for 24 h *in vitro* at 38.5°C under a 5%CO_2, in air, in TCM-199 medium supplemented with 0.5µg/mL FSH, 100 IU hCG/mL, and 1µg estradiol/mL. After 24 hours, the oocytes were fertilized in TALP with 10 µg/mL heparin (control), added with 50 (M 2-ME or 50 (M Cist or 5 mM buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, which facilitates ROS action. The semen was diluted at a concentration of 100x103/100 µL TALP. Sperm morphology was evaluated at 0 and 4 hours after the beginning of fertilization (hpi). Embryo cleavage was evaluated at 36 hpi and data were analysed through ANOVA (P<0.05). Cleavage rates were 73.1% (control), 67.6% (Cist), 46.4% (2-ME), and 68.6% (BSO), with no differences (p>0.05) observed among groups. The percentage of sperm with defects at 4 hpi were accepted as normal for frozen-thawed semen (less than 30%), being 15.7% (control), 19.1% (Cist), 25.4% (2-ME), and 10.6% (BSO). Even though no significant differences were found these data may suggest an apparent harmful effect of the 2-ME during the FIV. This effect, however, must be better evaluated.

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EFFECT OF IN VITRO FERTILIZATION PERIOD AND CAFFEINE ON REDUCTION OF POLYSPERMIC EMBRYOS IN PIGS

Oliveira, V.P.; Marques, M.G.; Nascimento, A.B.; Gonçalves, J.S.A.; Mendes, C.M.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil - purri@usp.br

In vitro production of pig embryos has high incidence of polyspermy as a major problem. Polyspermic embryos reach blastocyst stage, however the number of inner cell mass are lower, probably one of the reasons to explain the low or blocked *in vitro* embryonic development. The aim of this study was to evaluate three periods of *in vitro* fertilization (2, 4 or 6 hours) with refrigerated semen, capacitated with 2mM of caffeine during in vitro fertilization or pre-capacitated during 3 hours before fertilization on polyspermy reduction. Oocytes from pre-puberal gilts were matured for 44 hours in TCM199 and fertilized in MTBM medium using $1x10^5$spermatozoa/ml in three different periods (2, 4 or 6 hours). Eighteen hours after fertilization, embryos were fixed and stained with Hoechst 33342 and pro-nucleus were evaluated. The results were analyzed by SAS and PROC NPAR 1WAY for non parametric analysis of variance. The effect of treatment was verified by Kruskal-Wallis Test and multiple comparisons were done two by two by Wilcoxon Test, with significance level of 5%.The capacitated spermatozoa rates before or during fertilization at 2, 4 or 6 hours of IVF were 58.9 and 51.6%; 38.6 and 49.4% and 30.1 and 41.3% for non fertilized oocytes; 23.3 and 23.6%; 40.3 and 30.6% and 41.6 and 32.6% for monospermic embryos and 17.8 and 24.8%, 21.1 and 20.0% and 28,3 and 26.1% for polyspermic embryos. There was no difference among semen treated before or during fertilization on monospermic or polyspermic embryo rates. In conclusion, at these conditions, the reduction of fertilization time did not influence monospermic or polyspermic embryo rates.

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MITOCHONDRIAL ACTIVITY IN BOVINE SPERMATOZOA INCUBATED AT DIFFERENT PERIODS WITH OR WITHOUT EXOGENOUS DNA

Feitosa, W.B.; Rovegno, M.; Simões, R.; Milazzotto, M.P.; Caetano, H.V.A.; Visintin, J.A., Assumpção, M.E.O.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil - wfeitosa@usp.br

The aim of this work was to evaluate the effect of exogenous DNA on the mitochondrial membrane potential in bovine spermatozoa. The semen was thawed and centrifuged at 600g for 30 minutes in Percoll gradient (45/90%). The pellet was resuspended and washed 3 times in TALP-semen and centrifuged at 200 x g for 5 minutes. Spermatozoa were resuspended in IVF-medium (without heparin) at a concentration of 5 x 10^6cells/mL and incubated at 39°C and 5%CO_2, in air, with 500ng/mL of pEYFP-Nuc (Clonetech BD Biosciences,Franklin lakes, NJ-USA; DNA group) or without DNA (control group) for 4 hours. The mitochondrial membrane potential was evaluated after 0, 1, 2, 3 and 4 hours (h) of incubation, using the fluorescent probes JC-1 (Molecular Probes, Eugene, OR-USA), which stains mitochondria in red when membrane potential is high and in green when it is low. A volume of 50µL of IVF-medium with 5x10^6cells/mL were incubated with 2µLof JC-1 (76.5mM in DMSO) at room temperature for 15 minutes. Then, a total of 200 cells were counted under epifluorescent microscope (Zeiss). Statistical analyses were performed using SAS, Tukey test and $p<0.05$ taken as significant. The percentage of spermatozoa that had mitochondria with high potential membrane at different hours was 81.9%^{a} (0 h), 68.4%^{b} (1 h), 47.8%^{c} (2 h), 29.8%^{d} (3 h), and 20.6%^{d} (4 h) for the control group. In group with DNA, the mitochondria with high membrane potential was 81.9%^{a} (0 h), 69.2%^{b} (1 h), 46.9%^{c} (2 h), 27.4%^{d} (3 h), and 22.3%^{d} (4 h). No differences ($p>0.05$) were observed between control and DNA groups on the mitochondrial membrane potential of the spermatozoa at any period of incubation. In conclusion, the DNA did not influence mitochondrial membrane potential of bovine spermatozoa.

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INFLUENCE OF EXOGENOUS DNA ON BOVINE SPERM CAPACITATION

Feitosa, W.B.; Milazzotto, M.P.; Simões, R.; Nicacio, A.C.; Nascimento, A.B.; Visintin, J.A.; Assumpção, M.E.O.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil - wfeitosa@usp.br

Mammalian sperm cells have a natural ability to take up exogenous DNA, being used as a vector to the generation of transgenic animals, since they transfer foreign DNA into oocytes during the fertilization process. The aim of this work was to evaluate the influence of exogenous DNA on bovine sperm capacitation. Thawed semen was centrifuged at 600 x g for 30 minutes in Percoll gradient (45/90%) to separate live spermatozoa. Pelleted semen was resuspended and washed in TALP-semen medium and centrifuged at 200 x g for 5 minutes. Three treatments were tested with ten replicates each one. For all treatment groups, 5 x 10^6spermatozoa/mL were used in 1 mL of IVF-medium and incubated for 4 hours at 39°C and 5%CO_2 in air. Then, semen was incubated in IVF-medium without heparin (control group), in IVF medium with 500ng/mL of pEYFP-Nuc Vector (BD Bioscience, Franklin Lakes, NJ-USA; DNA group), and in IVF medium with 100µg heparin/mL (heparin group). Propidium iodide and carboxifluorescein diacetate fluorescent probes were used to evaluate sperm capacitation. For statistical analysis was applied the SAS, using Tukey test with significance level of 5%. No significant differences ($p>0.05$) were found among DNA group (17.5% ± 1.6^{ab}), heparin (24% ± 2.5^{a}) and control (16% ± 1.9^{b}) groups for capacitation rates, but differences were detected between heparin (24% ± 2.5^{a}) and control (16% ± 1.9^{b}) groups. In conclusion, the incubation of sperm cells with DNA does not influence the sperm capacitation, and could be an alternative method to produce transgenic embryos.

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EFFECT OF INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE ON MOTILITY AND MEMBRANE INTEGRITY OF BOVINE *IN VITRO* CAPACITATED SPERMATOZOA

Ferreira-Berbari, J.B.P.[1]; Caldas-Bussiere, M.C.[1]; Paes de Carvalho, C.S.[1]; Silva, A.E.[1]

Setor de Reprodução Animal, Laboratório de Melhoramento Genético Animal, CCTA, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, 28013-602, Brazil.
jbbarcelos@uol.com.br

The aim of this experiment was to evaluate the nitric oxide (NO) influence, synthesized by inducible nitric oxide synthase (iNOS) on motility and membrane integrity during in vitro capacitation *in natura* Guzerá breed bovine spermatozoa. Different concentrations of aminoguanidine were used (AG – 0.1; 0.01; 0.001 M), specific inhibitor of iNOS, during *in vitro* heparin capacitation, during 300 minutes kinetics. Sperm motility was subjective evaluated by optic microscope, and membrane integrity with 0.2% Trypan-blue color. Variance analysis and test "t" ($P<0.05$) were used to compare the different treatments. The concentration of 0,1 M of AG diminished motility since 15 minutes in relation to control (10±0.0 % e 73.3±5.2 %, respectively). This parameter was completely inhibited at 120 minutes in relation to control and other treatments ($P<0.05$). The connections of spermatozoa acrosoma (5-8) were observed since 15 and up to 300 minutes, inhibiting the progressive motility. Therefore, this characteristic was not observed when 0.01 and 0.01 M were used. Motility diminish was verified at 300 minutes when 0.01 M of AG was used (16.7±15.1%), in relation to control and 0,1 M of AG (40.0±8.9 e 0%, respectively). The reduction of the number of integrity spermatozoa was observed at 15 minutes ($P<0.05$), comparing with control (64.5±8.2 %) and 0.1 e 0.01 M of AG (38.8±7.7 % e 46.2±9.6 %, respectively). This fact was not observed at other time and treatments. This results demonstrate that NO, synthesized by iNOS is involved on bovine spermatozoa motility during *in vitro* capacitation, but not on membrane integrity.

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EFFECTS OF VOLTAGE-DEPENDENT CALCIUM ION CHANNEL BLOCKERS ON MAMMALIAN SPERMATOZOA IN *IN VIVO* AND *IN VITRO* FERTILIZATION*

Gabaldi, S.H.[1]; Esper, C.R.[2]; Tedesco, A.C.[3]; Wolf, A.[4]; Oliveira, J.A.[5]

[1] Dep. de Reprod. An. - FMVZ-USP, São Paulo, shgabaldi@hotmail.com; [2] Dep. de Med. Vet. Prev. e Reprod. An. - FCAV-UNESP, Jaboticabal; [3] Dep. de Química - FFCLRP-USP, Ribeirão Preto, Brazil; [4] Dep. de Reprod. An.e Radiol. Vet. - FMVZ-UNESP, Botucatu, Brazil
[5] Dep. de Ciências Exatas - FCAV-UNESP, Jaboticabal, Brazil

There are reports about voltage-dependent calcium ion channel blockers of anti-hypertension therapeutic use causing male infertility, because calcium ions are important for sperm capacitation and acrosome reaction. The main goal of this experiment was to verify the influence of *L*-type voltage-dependent calcium channel blockers: verapamil, nifedipine and diltiazem on *in vivo* and *in vitro* fertility in hamster spermatozoa. *In vitro* treatments were preformed in the Experiment I, in which: spermatozoa were capacitated in medium with 0, 10μM, 100μM or 1mM of the three calcium ion channel blockers and they were evaluated for sperm capacitation, hyperactivity and *in vitro* fertility. The sperm capacitation was evaluated by fluorescent chlortetracycline staining technique in 5 moments (in the collection, 1, 2, 3 and 4 hours of capacitation), and *in vitro* fertilization was examined after 6 hours by male and female pronuclear formation by Hoechst 33342 staining technique and epifluorescent microscopy. In the Experiment II, verapamil (40mg/kg/d), nifedipine (50mg/kg/d) or diltiazem (40mg/kg/d) was supplied to males twice per day, by oral administration, during 60 days, and after this period, drug effects on the animals were evaluated through *in vivo* fertility (pregnancy of 16 females and 8 males/group), sperm capacitation and hyperactivity (8 males/group) and *in vitro* fertilization (5 males/group; 1667 oocytes). In the Experiment III, the intracellular calcium concentrations were measured in spermatozoa in the presence of 50μM of calcium ion channel blockers, by Fluo-3AM fluorescent intensity for 720 sec. Results of Experiment I and II were analyzed by Chi-Square Test ($p<0.05$), and, of Experiment III, by ANOVA ($p<0.05$). As results, in the Experiment I, the antagonists presented dose-dependent effects, affecting sperm capacitation rate, hyperactivity and *in vitro* fertilization rate on treated spermatozoa. Experiment II showed that male hamsters of treatment groups presented lower sperm capacitation rate, hyperactivity and *in vitro* fertilization rate and there were fewer pregnant females, demonstrating a reducing male fertility in verapamil and nifedipine groups when compared to the control and diltiazem groups. In the Experiment III, the antagonists blocked extracellular calcium influx in spermatozoa (control group = 2.51μM; verapamil = 0.45μM; nifedipine = 0.46μM, diltiazem = 0.40μM). It is concluded that, there were calcium ion channels with sensibility to *L*-type voltage-dependent calcium ion channel blockers in the plasmatic membrane of spermatozoa, and these antagonists influenced negatively on spermatozoa motility and hyperactivity, sperm capacitation, acrosome reaction and *in vivo* and *in vitro* fertilization rates and, thus, *L*-type voltage-dependent calcium channel blocker therapy reduced the fertility in hamster males.

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INFLUENCE OF ELECTROPORATION VOLTAGE ON BOVINE SPERMATOZOA VIABILITY FOR *IN VITRO* EMBRYO PRODUCTION

Simões, R.; Binelli, M.; Nicacio, A.C.; Yamada, C.; Milazzotto, M.P.; Rovegno, M.; Visintin, J.A.; Assumpção, M.E.O.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil – resimoes@usp.br

The purpose of this work was to evaluate the influence of electroporation voltage on bovine spermatozoa viability to improve *in vitro* embryo production (IVP). Oocytes from slaughterhouses ovaries were *in vitro* maturated in TCM-199 medium with 10%FCS + FSH + HCG + E^2 + piruvate + gentamicin under 5% CO_2 in air and at 39°C, for 24hs. Semen was thawed in water at 37°C for 30 seconds and separated in Percoll gradient (45/90%) at 600g for 30 min. Sperm cells ($5x10^6$) were submitted to electroporation at different voltages (100, 500, 750, 1000 or 1500V) in quadruplicate. The capacitance (25μF) and time (12msec) were constant to all voltages. Non-electroporated spermatozoa were used as control group. Spermamtozoa were washed in Talp semen medium by centrifugation at 200g for 5 min at 25°C. Supernatant was removed and $1X10^5$spermatozoa were used to inseminate 20 mature oocytes/ microdroplet for 18hs. Presumptive zygotes were co-cultured in SOFaa medium with granulosa cell monolayer under high humidified atmosphere, at 39°C and 5%CO_2 in air. Blastocyst (%BL) and hatching (%BE) rates were measured, respectively, at 9 and 12 days after insemination. Data were analyzed by ANOVA. The independent variables were replicate and voltage. The %BL and %BE varied from 17.2 to 45.1% and 39.7 to 98.8%, respectively, among replicates ($p<0.03$). Both rates decreased as voltage increased ($p<0.04$). Blastocyst rates were lower when voltage were higher than 500V. In conclusion these results indicate that electroporation does not improve embryo IVP, but low voltage could be useful for transgenic embryo IVP, since this method can be used for DNA incorporation by the cell.

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INFLUENCE OF THE BREED OF BULL AND THE BREED OF COW ON THE RESISTANCE OF BOVINE EMBRYOS TO HEAT SHOCK IN VITRO, AT EARLY STAGES OF DEVELOPMENT

Eberhardt, B.G.[1]; Satrapa, R.A.[2a]; Capinzaiki, R.L.[1]; Barros, C.M.[1]

[1]Depto. de Farmacologia, IB-UNESP, [2]Depto. de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP. brunogiorno@yahoo.com.br

It has been shown that *Bos indicus* embryos are more tolerant to heat stress (HS) than embryos from *Bos taurus* breeds. Recently, it was suggested that the oocyte may play a more important role than the spermatozoa on the development of tolerance to HS. In the present study the influence of both oocyte and semen, on the resistance to heat shock at early stages of in vitro development, was assessed in *Bos indicus* (Nelore), *Bos taurus* (Holstein and Angus) and crossbred. In Experiment 1, Nelore (N) and crossbred (CB) oocytes were collected from slaughterhouse ovaries and fertilized with spermatozoa from two Nelore (N) and two Angus (A) bulls. Embryos were collected and randomly assigned to control (39ºC) or heat shock at 12, 48 or 96 hours post insemination (HS 12, 48 or 96 hpi; 41ºC for 12 h) treatments. The cleavage rates and proportion of embryos developing to the blastocyst and hatched blastocyst stages were recorded on day 2, 8 and 10, respectively. HS 12, 48 and 96 hpi did not decrease cleavage rates in any group ($p>0.05$). However, HS 12 and 48 hpi reduced the percentage of embryos from Nelore and crossbred oocytes fertilized with Nelore or Angus bull that developed into blastocyst (NxN=47.8 vs. 35.3, NxA=52.6 vs. 35.7, CBxN=62.8 vs. 30.2 and CBxA=57.5 vs. 34.8; $p<0.05$, 12 hpi; NxN=61.6 vs. 51.7, NxA=61.1 vs. 44.4, CBxN=55.7 vs. 44.0 and CBxA=55.8 vs. 40.5; $p<0.05$, 48 hpi, where the first letter is the breed of the cow and the second the breed of the bull), but did not decrease blastocyst rates in any group at 96 hpi, with the exception of CBxA (NxN=39.1 vs. 34.9, NxA=45.8 vs. 40.2, CBxN=43.5 vs. 36.5, $p>0.05$; CBxA=45.0 vs. 34.3, $p<0.05$; 96 hpi). In Experiment 2, oocytes from Nelore and Holstein cows were fertilized with semen from bulls of either Nelore or Angus breeds, and subjected to 12 h HS at 96 hpi. Heat shock 96 hpi decreased blastocyst only in groups in which Holstein oocytes were used (HxA=27.9 vs. 12.7 and HxN=30.8 vs. 22.7; $p<0.05$, 96 hpi). There was a breed of cow x breed of bull interaction, HxN embryos were more resistant to heat stress than HxA (30.8 vs. 22.7 and 27.9 vs. 12.7, control vs. HS, $p<0.05$). It is concluded that Nelore embryos are more resistant to heat shock than Holstein or crossbred, at early stages of in vitro development. Additionally, the resistance to heat shock was a result of the genetic contribution from the oocyte and spermatozoa, since Holstein oocytes were significantly more resistant to heat shock (12 h, 96 hpi) when fertilized with Nelore as compared to Angus semen.

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DIFFERENCES ON *IN VITRO* PRODUCTION OF EXPANDED BLASTOCYSTS, BLASTOCYSTS AND INITIAL BLASTOCYSTS OF 29 BULLS TESTED.

Elias, F.P.[1]; Fernandes, M.B.[1]; Queiroz, L.F.M.[1]; Vila, R.A.[1]; Galerani, M.A.V.[1]; Nomelini, J.[1]; Lobo, R.B.[1]

[1]Departament of Genetics – FMRP/USP, Ribeirão Preto – SP, Av. Bandeirantes, 3900, 14040-030, Brasil. fpelias@genbov.fmrp.usp.br

The chance of a bovine embryo produced in vitro, to result in pregnancy after embryo transference (ET), depends among other factors, of the your´s development stage at the time of transference (Dayan, A., Theriogenology, vol. 57, p. 242, 2002). In this manner, the aim of this study was evaluate the production in vitro of embryos, expanded blastocysts (Bx), blastocysts (Bl) and initial blastocysts (Bi), using 29 bulls of the Programmed Reproduction from Program Nelore Brazil – USP. Briefly, the oocytes colleted from slaughterhouses (N=4.666) were matured in TCM199 + 10%, SBF + 0.5 μg, FSH/ml + 5μg, LH/ml + 1μg Estradiol/ml, during 24 hours at 38.5°C in 5% CO_2 in air atmosphere. Viable spermatozoids were obtained by centrifugation in Percoll gradient (45 and 90%), and used for in vitro Fertilization in a concentration of 2 million sptz/ml in TALP+10 μg de Heparin/ml medium. After 18 hours, the presumptive zygotes were transferred to a CR_2+10% SFB medium and co-cultured with *cumulus* cells. In the 4° day of cultivation the embryos with number of cells inferior to 16 were removed and medium of cultive was added. After 163 hours of the *in vitro* Fertilization, all embryos were classified in expanded blastocysts (Bx), blastocysts (Bl) and initial blastocysts (Bi). The Qui-square test and Coefficient of Correlation of Spearman were made by the Procediments Freq and Corr (HEALTHY Online Doc. Version 8. HEALTHY Inst. Inc., Cary, NC, USES, 1999), respectively. There is significant difference ($p < 0.01$) among bulls for *in vitro* Fertility, proportions of embryos in the stage of Bx and Bi. There is correlation between the *in vitro* Fertility and the production of embryos in the stage of Bl ($rs=0.82$; $p < 0.01$) and of Bx ($rs=0.40$; $p=0.03$), in relation to the total number of fecundated oocytes, but not in relation to the total number of embryos. Than animals of same *in vitro* Fertility can produce different proportions of Bx and Bi in relation to the number of total embryos and consequently, different pregnancies rates. The information of the *in vitro* Fertility of bull associated its acting in the production of embryos in the stage of Bx is of great interest for selection nuclei that use the system of *in vitro* production of embryos, have seen that this development stage possesses larger pregnancy chance after the transfer of the embryo.

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BULL VARIATION ON IN VITRO EMBRYO PRODUCTION AND INTERACTION WITH DIFFERENT DONOR COWS

Accorsi, M.F.[1]; Gonçalves, D.D.[1]; Ferraz, M.L.[1]; Watanabe, M.R.[1]; Meirelles, F.D.P.[2]; Meirelles, F.V.[2]; Watanabe, Y.F.[1,2]

[1]Vitrogen® Pesquisa e Desenvolvimento em Biotecnologia da Reprodução, Cravinhos–SP, Brasil.
[2]Depto de Ciências Básicas da FZEA-USP, Pirassununga-SP, Brasil. monica@vitrogen.com.br

In vitro embryo production (IVP) has been routinely used in large scale for commercial purposes, using animals of high zootechnical value. In the IVP process a variation in production in different animals (donors and bulls) can be observed. The aim of the present work was to assess embryo production and pregnancy rates of the most commercially used bulls and, consequently, the interference of donors in the process. Oocytes were obtained by ovum pick up (OPU) with the aid of a vaginal transducer connected to an ultrasound, and selected according to morphology for maturation. During IVM the oocytes remained 22-24h in TCM199 medium added with FSH/LH/Estradiol + 10% FCS at 38.8°C under 5% CO_2 in air. After this period, the matured oocytes were placed in IVF medium (TALP added with BSA, PHE and heparin 10μg/ml) containing $1x10^6$ motile sperm cells/ml, which were prepared by Percoll gradient (45 and 90%). After 20-22 h the zygotes were cultured in droplets of CR2 medium + 10% FCS in co-culture with granulosa cells. Only embryos that developed to the morula and blastocyst stages and of good quality were transferred to properly synchronized recipients. Nine bulls used with 139 donors in 1,021 OPU sessions were considered, where 16,800 viable oocytes and 5,929 embryos (35.3%) were obtained, from which 5,437 were transferred resulting in 1,977 pregnancies (36.4%). Data were analyzed by ANOVA and the means compared by TUKEY-KRAMER HSD test. According to the results a highly significant variation ($P<0.0001$) was observed among bulls regarding embryo (27.5±1.5% to 44.4±2.2%), and pregnancy rates (25.8± 1.7% to 48.7±1.9%), as well as mean pregnancies per bull at each OPU session (1.6±0.1 to 4.1±0.3). Analyzing some matings it was possible to observe that donors were a source of variation for the same bull. Bull F showed a pregnancy rate of 27.7% (467/1,507) and was mated with 36 donors in 287 replicates, presenting a variation from 0 (0/22) to 55.4% (46/83). The same occurred with bull A, mated with 6 donors in 31 replicates, with a mean pregnancy rate of 43.2% (189/282), varying among donors from 17.6 (6/34) to 60.7% (21/34). In conclusion, there was a significant difference among bulls regarding embryo production, pregnancy rates and mean pregnancies per OPU session. Besides this, the donors interfered significantly in the production of the same bull, assuming the interaction donor x bull as an important influence on the results of commercial *in vitro* embryo production.

BULL EFFECT AT THE *IN VITRO* EMBRYO DEVELOPEMENT RATE DELIVERED FROM OVUM PICK-UP GUIDE BY ULTRASOUND

Morelli Júnior, J.[1]; Atique Netto, H.[1] ;Martins, A.R.[1]; Prado, R.B.[1]; Fazio, F.L.[1]

[1] Centro Universitário de Rio Preto – Hospital Veterinário "Dr. Halim Atique", UNIRP, São José do Rio Preto - SP, 15025-400, Brasil. halimnetto@unirpnet.com.br

The embryos *in vitro* production (IVP) is a technique widely used commercially and including in oocytes maturation and fecundation studies, spermatic ability, embryos culture, cloning, embryos sexing, gene transfer, spermatozoa microinjection, spermatozoa and in oocytes and embryo freezing and thawing techniques. When some adjusts on the IVP technique are made, there are differences between the bull fecundation rate, blastocyst development, eclosion and consequently in the ability of these embryos (SHI et al., 1990; KREYSING et al., 1997; ACCORSI, 2000). Spermatozoa of frozen and thawed semen are wide used in programs of IVP due its viability and practical convenience (LEIBFRIED-RUTLEGE, 1999). The main objective of this study was to evaluate the effect of different bulls in the production of *in vitro* embryos (IVE). Sixteen Nelore bulls semen were used, they were tested in relation to the fertility in the field (artificial insemination) commercialized in central offices duly regularized by the ASBIA (Brazilian Association of Artificial Insemination, Uberaba – MG, Brazil) and submitted to *in vitro* fertilization. The 54 cows downer used belong to Mata Velha Farm (BR 0,50, km 192), located in the city of Uberaba – MG, Brazil, with age range from 25 to 218 months and with fertility proven in the property (anamnese and gynecological examination). The ovum pick-up guided by ultrasound (OPU) was made with an ultrasound device (aloka SSD-500V) and with a convex probe of 5MHz connected to a vacuum bomb (cook - 75 mm Hg), the cows had not been submitted to the application of gonadotrofins. After each session of aspiration, the oocytes were selected by its morphology and immediately sent to the laboratory where the *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) were made. A percentage of 7.69% (lowest rate) of embryos to bull 01 was seen, with 39 oocytes, 13 usable oocytes, which developed 1 embryo and 65%(highest rate) to bull 16, with 28 oocytes, 20 usable oocytes, which developed 13 embryos. An 42.49% average between *in vitro* fertilization and the embryo production rate was seen. Qui-square test ($32.83>30.57$), $gl=15$; $p<0.01$, demonstrate a significant difference among the bulls. This study shows how bulls selection are important for usage on embryos *in vitro* production programs.

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EFFECT OF FOLLICULAR ASPIRATION PRESSURE ON RECOVERY RATE AND CUMULUS-OOCYTE COMPLEX QUALITY IN GOATS AND EWES EXPLORED IN THE NORTH-EASTERN BRAZIL

Paula, N.R.O.; Magalhães, D.M.; Arruda, I.J.; Andrade, M.L.L.; Souza, A.L.; Pereira, A.F.; Almeida, K.C.; Avelar, S.R.G.; Silva, J.D.A.; Rondina, D.; Freitas, V.J.F.

Laboratory of Physiology and Control of Reproduction - FAVET/UECE, 60740-000, Fortaleza-CE, Brazil. neyromulo@uece.br

Follicular aspiration is the first step of *in vitro* embryo production (IVP) process. Thus, the success of follicular aspiration implicates in a larger number of recovered viable structures, resulting in the improvement of *in vitro* maturation, fertilization as well as the embryo culture. However, there is a paucity of information concerning the determination of an ideal follicular aspiration pressure destined to IVP in small ruminants. This study aimed to verify the effect of the pressure exercised in vacuum pump during follicular puncture on the recovery rate and cumulus-oocyte complexes (COC's) quality in goats and ewes without defined race (WDR) explored in the North-eastern Brazil. The experiment was carried out in the Laboratory of Physiology and Control of the Reproduction (FAVET / UECE). Fifty-four pairs of ovaries were obtained at a local slaughterhouse from WDR ewes (27 pairs) and goats (27 pairs) explored in the semi-arid of the Ceará State. The ovaries were transported to the laboratory in thermal container containing saline solution at 37 ºC. At the laboratory, the ovaries were transferred to another container in water-bath containing new saline solution at 37 ºC. Follicular puncture was accomplished with a vacuum pump (GenX international, Pioneer Pro-Pump, USA) attached to an 18 G needle. For each species, the ovaries were equally divided in three groups of different pressures (50, 100 and 150 mmHg). After follicular puncture, the flushing was poured into a Petri dish and was observed under a stereomicroscope (Nikon, SMZ-1B, Japan). The recovery rate (number of COC's/ovary) was observed, as well as the quality degree of COC's: degree I (oocyte surrounded by three or more cumulus cells layers); degree II (oocyte surrounded by one to two cumulus cells layers); degree III (denude oocyte) and degree IV (degenerate oocyte or with broken zona pellucida). The efficiency of different pressures was compared using the Chi-square test ($P < 0.05$). In goats, it was obtained a larger number of recovered structures at 100 (n=93) and 150 (n=79) mmHg pressures, being verified mean recovery rates of 3.22, 5.17 and 4.39 for 50, 100 and 150 mmHg pressures, respectively. Concerning the COC's quality, structures of degree I were observed only in 50 mmHg pressure and a larger number of structures of degree II was verified in this pressure. In ewes, mean recovery rate was of 2.00, 2.22 and 1.83 for 50, 100 and 150 mmHg pressures, respectively. In addition, a larger number of poor COC's was observed in 150 mmHg pressure. Therefore, for WDR goats and ewes explored in the North-eastern Brazil, 50 mmHg pressure demonstrated to be ideal for obtaining viable COC's to be used in subsequent steps of *in vitro* embryo production.

FOLLICULAR ASPIRATION IN GOAT FEMALES THROUGH LAPAROSCOPY TECHNIQUE

Cordeiro, M.F.[1]; Vicente, W.R.R.[1]; Di Filippo, P.A.[1] ; Alves, A.E.[1]; Ribeiro, G.[1]; Pavão, G.D.[1]; Dias, D.P.M.[1].; Dória, R.G.S.[1]; Beretta, C.A.G.[1]; Pereira, R.F.S.[1]; Sousa, R.A.F.[1]

Department of Animal Reproduction - FCAV/UNESP, Jaboticabal - SP, 14884-900, Brazil.
wilter@fcav.unesp.br

The laparoscopy has gained prominence amongst the surgical techniques for oocytes recovering in small ruminants. Due to be less invasive and has lower time of execution this procedure reduces animal's stress (BALDASSARRE *et al*., 2002, Theriogenology, v.57, p.275-284; KÜHHOLZER *et al*., 1997, Theriogenology, v.48, p.545-550). This work aimed to evaluate the follicular aspiration performed by laparoscopy technique in adult and young goats who were submitted to repeated interventions. Oocytes of six goats had been harvested (three adults and three young) once a week during six weeks resulting in 36 sessions. Three punctures at the abdomen (cranialy to the udder) introduced trocars that arranged endoscope, non-traumatic grasping forceps and needle system aspiration connected to a vacuum pump. CO_2 was insufflated using a total pressure of 6 to 8mmHg for young goats and 10 to 12mmHg for the adults. The needle system aspiration was introduced inside the cavity through a plastic tube due to avoid perforation risks of abdominal organs. The ovary was fixed with non-traumatic grasping forceps and tip of the needle was displayed only when the aimed follicle was close. At the end of aspiration session, ovaries were washed with heparinized PBS solution in order to prevent presumptive adhesions. Follicular fluid aspiration was collected in filters and oocytes were recovered under stereomicroscope, classified according to their quality and transferred to maturation medium under mineral oil, during 27 hours, 39°C, 5,0% of CO_2 in air and saturated humidity. When culture ended, all oocytes had been fixed for posterior observation of nuclear maturation stage. The average of total oocytes number recovered was 26/donor. Recovery rate was considered low at young (26.15%) and adult (42.65%) group which can be attributed to punctured follicles number that were inferior than those observed follicles, as well as the needle diameter (18G) avoiding the aspiration of small follicles (<5mm). The pressure used denuded some oocytes compromising its quality. However, maturation rate was considered satisfactory wich didn´t show difference between young (64.3%) and adult (78.5%) groups. At the majority females no adherences were observed between genital and abdominal organs. Skin punctured had healed at the third day. During the experiment the practitioner experience and team harmony reduced the time of laparoscopy sessions which was in average 35 minutes. Laparoscopy technique for follicular punctured is safe and it can be performed at the same donor as far as once a week.

EFFECT OF LH ON QUALITY OF OOCYTES OBTAINED BY OPU IN GUZERÁ BREED ANIMALS

Ueno, V.G.[1]; Fontes, R.S.[1]; Fonseca, F.A.[2]; Gama Filho, R.V.[2]; Bucher, C.H.[1]; Ramos, J.L.G.[1]; Carvalho, C.S.P.[1]; Shimoya, A.[3]

[1]Laboratório de Melhoramento Genético Animal, UENF, Campos-RJ, Brasil, [2]Laboratório de Zootecnia e Nutrição Animal, UENF, Campos-RJ, Brasil; [3]Laboratório de Estatística, Universo, Campos-RJ, Brasil vanessaueno@hotmail.com

The present experiment aimed to evaluate the effect of LH in the quality of oocytes obtained from ≤ 6mm e > 6 mm follicles by OPU in Guzerá cattle. All animals (n=8) were synchronized by insertion of a progesterone-releasing device (CIDR-B®), during of nine days, together with 2 mg estradiol benzoate (Estrogin®) injection, at any phase of the estrus cycle considered day zero (D0). Five days after the device insertion (D5), the stimulatory treatment was initiated with a total dosage of 100 mg FSH (Folltropin-V®) during three consecutive days, with decreasing dosages and intervals of 12 hours between the applications, 48 hours after the stimulatory initiation of treatment an intramuscular injection of 500 μg PGF2α (Ciosin®) was given. In group I (control, n=4), the animals received only FSH and, in group II (LH, n=4) the FSH administration was given together with an intramuscular application of 25 mg LH (Lutropin®) 6 hours before the OPU. In both groups the OPU was accomplished 48 hours after the last FSH application. All data were submitted to ANOVA and means compared by the Tukey test. Could be observed significative difference between the follicles sizes in the group LH, with total of oocytes and e number of oocytes degrees I and II deriving from follicles ≤ 6mm (16 ± 3.82 and 8.0 ± 1.61) and > 6 mm (7.3± 4.61 and 3.7± 1.5). In relation to the control-group were not observed significative differences between follicle sizes ≤ 6mm (8.3 ± 5.18 and 3.5 ± 1.20) and > 6 mm (6.0± 2.94 and 2.5± 1.0) in the total of oocytes and in the number of oocytes degrees I and II. With the results obtained in this work, can be suggested that the used protocol associated to an LH injection provided the increasing in the number and in quality of oocytes degrees I and II.

EVALUATION OF FSH ADMINISTRATION BEFORE OVUM PICK-UP ON OOCYTES RECOVERY AND *IN VITRO* EMBRYO PRODUCTION IN NELORE (*Bos taurus indicus*) COWS

Nonato Junior, I.[1]; Pontes, J.H.F.[1]; Ereno Junior, J.C.[1]; Sanches, B.V.[1]; Seneda, M.M.[2]

[1] In Vitro Brasil, Mogi Mirim-SP, 13800-970, Brasil. [2] Departamento de Clínicas Veterinárias – CCA/UEL, Londrina-PR, Brasil. ismael@invitrobrasil.com.br

Now days embryo production is in expressive growth, due to the commercial use of reproductive biotechnologies, mainly in South of America, more specifically in Brazil (REINCHENBACH, *Acta Scientiae Veterinariae*, v.31, p.15-27, 2003). The aim of this study was evaluate the effects of FSH administration before ovum pick-up on *in vitro* embryo production in Nelore cows. Donor animals (5) were submitted to 26 ultrasound guided transvaginal ovum pick-up sessions performing at control group (CG) and a treatment group (TG). All donors were aspirated in both groups. During the treatment 3 mg Norgestomet implant were placed in non-cycling donors at day 0 (D0), and the dominant follicles were aspirated at day 3 (D3). Among day 4 (D4) and day 6 (D6) 50 mg FSH (Folltropin, Vetrepharm, Belleville, Canada), i.m., were given in two doses with in 12 hours, and after 36 hours, the implant was removed and the ovum pick-up was performed. The mean umber of oocytes recovered was 29.30 in the CG, and 24.90 in the TG. The rates of embryo production were 28.90% in CG and 39.0% in TG. The use of FSH before ovum pick-up didn't increased the number of oocytes obtainment, although shave contributed to increase embryo production rates, what is beneficial to *in vitro* embryo production.

INFLUENCE OF HEAT STRESS ON BLASTOCYST RATE OF BOVINE EMBRYOS (*Indicus* vs *Taurus*) FROM OOCYTES OBTAINED BY OVUM PICK UP (OPU)

Sartorelli, E.S.[1a]; Barcelos, A.C.[2b]; Satrapa, R.A.[1a]; Nogueira, M.F.G.[1a]; Martins, D.F.[2]; Eberhardt, B.G.[1a]; Potiens, J.R.[3]; Seneda, M.M.[4]; Barros, C.M.[2]

[1] Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP; [2] Departamento de Farmacologia, IBB/UNESP, Botucatu-SP, 18618-000, [3]Central Bela Vista (Pardinho, SP), [4]Departamento de Clinicas Veterinárias, CCA, Universidade Estadual de Londrina, PR, 86051-990, Brasil. essartorelli@yahoo.com.br

There are evidences that the deleterious effects of heat stress on fertility are less pronounced in *Bos indicus* than in *Bos taurus* breeds, due primarily to differences in their thermoregulatory capacity. In the present work the resistance to heat stress of Nelore embryos (*Bos indicus*) was compared to *Bos taurus* breeds non adapted (Angus) or adapted (Bonsmara) to high temperatures. In experiment (exp.) 1 (Nelore vs Angus) and 2 (Nelore vs Bonsmara), oocytes obtained by OPU were matured (TCM-199), fertilized and culture (SOFaaci) *in vitro*. Ninety six hours pos-insemination (hpi) embryo with more than 16 cells were randomly allocated in two main groups: Group Control (embryos were maintained at 39 °C all the time) and Group HS (embryos were maintained at 41 °C during 12 h and afterwards returned to 39 °C). Blastocyst rates were determined on the 7th day of culture. In exp. 1, 294 oocytes from Nelore and 144 from Angus cows had a cleavage rate of 67.9 and 59.4%, respectively. Ninety six hpi embryos (> 16 cells) were distributed in 4 groups: Nelore Control (n=97), Nelore HS (n=95), Angus Control (n=34) and Angus HS (n=25). The blastocyst rates were 39/97(40.2%), 23/95(24.2%), 19/34(55.9%) e 8/25(32.0%), respectively. The decline on blatocyst rate caused by heat stress on Nelore (16.0%) and Angus (23.9%) were close to significance (ANOVA, p=0.069), and indicates that Nelore embryos may be more tolerant to HS than Angus embryos, from oocytes obtained by OPU. However, it is necessary to increase the number of blastocysts per group in order to better characterize the effects of heat stress on these embryos. In exp. 2, 294 oocytes from Nelore and 101 from Bonsmara cows had a cleavage rate of 41.2 and 51.2%, respectively. Ninety six hpi embryos (> 16 cells) were distributed in 4 groups: Nelore Control (n=44), Nelore HS (n=49), Bonsmara Control (n=22) and Bonsmara HS (n=22). The blastocyst rates were 35/44 (79.5%), 30/49 (61.2%), 10/22(45.5%) e 6/22 (27.3%), respectively. In spite of the fact that Bonsmara had a lower blastocyst rate as compared to Nelore, the decline on blastocyst rate caused by HS was very similar in Nelore (18.3%) and Bonsmara embryos (18.2%). Additional OPU are underway to test the hypothesis that thermotolerance of Nelore embryos are similar to embryos from a *Bos taurus* breed adapted to high temperatures (Bonsmara), and superior to embryos from a non adapted breed (Angus).

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[b]CAPES.

USE OF COCONUT WATER SOLUTION AS PRESERVATION MEDIUM FOR THE TRANSPORT OF IMMATURE BOVINE OOCYTES

Cordeiro, M.S.[1]; Silva*, E.H.S.[1]; Biondi, F.C.[1]; Miranda, M.S.[1]; Pauxis*, D.R.[1]; Santos, S.S.D.[2]; Andreoti, M.[1]; Ohashi, O.M.[1]

[1] Laboratório de Fertilização *in vitro* - CCB/UFPA, Belém-PA, Brasil; [2] Departamento de Histologia e Embriologia - CCB/UFPA, Belém-PA, Brasil; * Bolsista PIBIC - CNPq
marcela@ufpa.br

The development of a Preservation medium which can preserve the oocyte viability during the transport is an important step to the commercial applicability of PIV. The stress caused by the conditions and time of transport became one of the limiting factors on the maintenance of oocyte quality. Coconut water solutions have been used for preservation of semen, maintenance and culture of cells and tissues of many species in attempt to preserve celullar viability. This work aims to evaluated a coconut water solution as a holding medium to be used on the transport of bovine imature oocytes in PIV programs. To simulate the transport, bovine oocytes from slaughterhouse were selected and distributed in: Control Group - oocytes submitted to IVM immediately after the selection; Group 1 - oocytes maintened in TCM 199 with 10% FCS, antibiotics and pyruvate, during 6, 9 and 12 hours, protected from light at 30ºC; and Group 2 - oocytes maintened in a coconut water solution (75% of coconut water + 25% of ultrapure water) with 25 mM Hepes, 10% FCS, antibiotics and pyruvate, during 6, 9 and 12 hours, under the same conditions for Group 1. After each time, the oocytes were maturated in TCM199 bicarbonate with 10% FCS, hormones, insulin, 2 mercaptoethanol, pyruvate and antibiotics, in 5% CO_2 at 38.5ºC with humid air, during 18 hours. After IVM, the oocytes were *in vitro* fertilized and cultured in CR2 medium. The cleavage, blastocyst and hatching rates were analysed on the 2^{nd}, 7^{th} and 9^{th} day of culture, repectively. Comparisons between ratios were performed by chi-square test, and the differences were considered to be significant at $p < 0.05$. The data analysis showed that when compared Group 1 and Group 2, the only statistic difference was at 12 h, where the oocytes exposed to coconut water solution (Group 2) had higher blastocyst rate (49.18% *vs.* 26.67%). To determine if the viability was damaged by the time of transport, Groups 1 and 2 were compared with the Control Group. In this comparision, the Group 2 did not differ in any rate, but in Group 1, there was difference, after 9 h of transport, for cleavage and blasocyst rates (50.85% *vs.* 75% e 25.42% *vs.* 53.33%) and after 12 h of transport, only for blastocyst rate (53.33% *vs.* 26.67%). In according with this results, the coconut water solution can be used in the maintenance of bovine imature oocytes during 12 hours with low damage of oocyte viability.

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INFLUENCE OF THE YEAR SEASON IN THE OOCYTES PRODUCTION IN NELORE COWS

Rubin, K.C.P.[1]; Max, M.C.[1]; Pontes, J.H.F.[2]; Nonato Jr., I.[2]; Ereno Jr., J.C.[2]; Uvo, S.[2]; Sanches, B.V.[2]; Rocha, J.L.[3]; Seneda, M.M.[1]

[1]Departamento de Clínicas Veterinárias, CCA, Universidade Estadual de Londrina, PR, 86051-990. karinac8@aol.com; [2] In Vitro Brasil – Central de Biotecnologia da Reprodução – Mogi Mirim; [3] IAPAR – Instituto Agronômico do Paraná – PR.

The objective of the present work was to analyze the influence of the year season in the ovocytes production in cows of Nelore breed submitted to the follicular aspiration (FA). Ninety six sessions of FA were realized in 30 Nelore donors, nonpregnants, and with average age of five years. In average 3,2 aspirations were done in each animal, at least one and in maximum five, in an approach period of 4 months. From the 96 collections were obtained 2463 ovocytes. Among these, 2192 ovocytes were classified as viable (GI, GII, GIII) and 263 as unviable ones. During the spring and summer period were realized 54 sessions and 42 sessions were realized in the autumn and winter period. In the spring and summer period the average of the ovocytes were 25.77 per session, of which 94.30% were viable. In the autumn and winter period were obtained the average of 26,12 ovocytes per session and 83.06% of them were viable. In the analyzed periods (spring/summer or autumn/winter), there were no significant differences ($p>0.05$) between the average ovocytes production. On the other hand, there was found a significant difference ($p\leq0.05$) in the percentage of the viable ovocytes; the best percentage was obtained in the spring/summer period. These results suggest an increment in the efficiency of the ovocytes obtained with the follicular aspiration procedures concentration, during the hottest months of the year, since there is a bigger possibility of achieving ovocytes with a better quality for *in vitro* produced embryos. Other aspects, as the availability of forages and the offer of recipients tend to be facilitated in the same spring/summer period, strengthening the optimization perspective of the *in vitro* produced embryos in this period.

IN VITRO MATURATION OF TRANSPORTED EQUINE OOCYTES.

Jáuregui Lorda, M.[1]; Herrera, C.[3]; Pinto, M.[1]; Losinno, L.[2]; Miragaya, M.H.

[1]Area de Teriogenología. Facultad de Ciencias Veterinarias. Universidad de Buenos Aires, Argentina; [2]Cátedra de Producción Equina, Facultad de Agronomía y Veterinaria. Universidad Nacional de Rio Cuarto, Rio Cuarto, Argentina; 3. Halitus Biotecnología, Halitus Instituto Médico, Buenos Aires, Argentina

Optimal conditions for *in vitro* maturation equine oocytes are still not well defined. There is limited information about energy supplies and hormones requirements for *in vitro* maturation of equine *cumulus-oocytes-complexes* (COCs). This is important for further *in vitro* embryo production by intracytoplasmic sperm injection (ICSI) technique. Therefore, *in vitro* embryo production would be increased using this technique to fertilize oocytes since *in vitro* fertilization (IVF) has not been successful as in other species. The aim of this study was to mature equine oocytes *in vitro* obtained from slaughterhouse ovaries and transported in a container under controlled temperature. Ovaries were transported at 25°C in saline solution to the laboratory within two hours after mares were slaugthered. Every follicle between 7 and 30 mm was individually dissected from the ovary and the follicle walls were scraped and washed with heparinized Ringer Lactate solution. COC's were located with a stereomicroscope and were classified as compact, expanded and denuded *cumulus* cells. They were transported in a shipper at 38°C for 8 hours, 600 Km in culture media TCM199 with Hepes. The mares ovaries processed were 503 and 1137 follicles were obtained, and 904 oocytes recovered (79.5% oocyte recovery rate from follicles), 364/904 had compact *cumulus* cells (40.2%), 377/904 expanded (41.7%) and 163/904 were denuded (18.0%). TCM 199 with FSH 5μg/ml, LH 2μg/ml, IGF-1 100μg/ml, EGF 100ng/ml, and 10% FCS at 38°C in 5% CO_2 in air was used as in vitro maturation medium. Compact COC's were cultured for 36 h and expanded COC's for 24 h. Cumulus cells were removed with 10% hialuronidase in PBS and those which presented a visible polar body were considered matured. A total of **220** oocytes were placed in culture after arrival (**99** compact COC's and **121** expanded COC's) .The percentage of metaphase II oocytes were 84.0%, (185/220) 41.0% (76/185) from compact and 58.9% (109/185) from expanded . These results show a high rate of *in vitro* maturation using this system. Therefore oocytes transported in a shipper under controlled temperature could be used for in vitro maturation programs. These would represent an improvement in the conditions for future assisted reproduction techniques.

DEVELOPMENTAL EVALUATION OF *IN VITRO* PRODUCED EMBRYOS FROM OOCYTES EXPERIMENTALY EXPOSED TO BOVINE HERPESVIRUS TYPE 1 (BHV-1) DURING MATURATION

D'Angelo, M.; Athayde, C.S.; Souza, R.J.; Vieira, S.E.M.; Malaguti, J.M.A.; Rojas, N.; Melo, G.M., Pavão, D.L.

Centro de P&D de Sanidade Animal do Instituto Biológico de São Paulo, Av. Conselheiro Rodrigues Alves, 1252, 04014-002- São Paulo, BR dangelo@biologico.sp.gov.br

The BHV-1 causes infectious bovine rynotracheitis (IBR), and can lead to abortion and embryo reabsorption. Due to the increase of *in vitro* embryo production, the necessity of studies for embryo sanitary control arose. The aim of this study was to evaluate the development of *in vitro* fertilized embryos derived from BHV-1 exposed oocytes during *in* vitro maturation. Morphological and developmental changes of these embryos, after *in vitro* fertilization, were evaluated. Oocytes (n=264) were aspirated from ovaries of slaughtered cows and they were randomly distributed into control (n=131) and exposed (n=133) to 30µl of BHV-1 suspension (Colorado strain, ATCC-VR864, $10^{4,5}TCID_{50/mL}$ titer) groups. Oocytes were maturated during 24 hours and fertilized in HTF medium, supplemented with 10% bovine fetal serum. After 5 and 6 days, embryos were evaluated and photographs were taken under inverted optical microscope. The preliminary results of the study showed that on day-5, 0.75% (n=1) of the presumptive zigotes from infected group reached the 2-cell stage, and the other structures showed morphological alterations such as grained and retracted cytoplasm and raised perivitelline space. In the control group, the clivage rate was 45.0% (n=59), with 29.5% (n=39) of the embryos reaching 8 cells, 10.5% (n=14) morula stage and 5.0% (n=6) 2 cells. On day-6, in the control group, embryos developed to morula stage (38.9%; n=51). These results probably indicate that BHV-1 could affect *in vitro* embryo fertilization and/or development,. The prominence of this study is to elucidate pathogens/gametes interaction, aiming the improvement on prevention of diseases dissemination by artificial reproductive techniques in animals.

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NUCLEAR MATURATION EVALUATION OF OOCYTES FROM ESTRUS AND ANOESTRUS BITCHES USING TWO CULTURE MEDIA.

Martins, L.R.[1]; Ponchirolli, C.B.[1]; Beier, S.L.[2]; Landim-Alvarenga, F.C.[1]; Lopes, M.D.[1]

[1]Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ Botucatu, Universidade Estadual Paulista, denise@fmvz.unesp.br; [2] Departamento de Anestesiologia – FM Botucatu, Universidade Estadual Paulista.

The present study aimed to evaluate the nuclear maturation of oocytes originating from estrus and anoestrus bitches. Two culture media were used, TCM 199 (*Tissue Culture Medium*) and SOF (*Synthetic Oviduct Fluid*). The females were divided into two groups: group 1, consisting of eleven (11) anoestrus bitches, and group 2, consisting of six (06) estrus bitches. In each group, oocytes were harvested from sliced ovaries and half of them were immediately stained with Hoechst 33342. The oocytes were classified as in GV (germinal vesicle), GVBD (germinal vesicle breakdown), MI (metaphase I) and D/NI (degenerated/non identifiable) and the other part was matured in SOF or TCM media for 24 hours, stained and evaluated as described previously. There were 511 and 373 grade I oocytes recovered in groups 1 and 2, respectively. The mean number of oocytes obtained per anoestrus bitch was 46.45 ± 25.23. For females in group 2, this value was 62.16 ± 8.18 oocytes / bitch. Oocytes from estrus and anoestrus bitches submitted to *in vitro* maturation presented similar rates of GVBD (21.68% TCM 199 and 23.61% SOF – anoestrus; 20% TCM199 and 23% SOF - estrus). However the MI rate was higher in the estrus group (9.79% TCM 199 and 9.72% SOF – anoestrus; 25% TCM 199 and 58% SOF – estrus). The protocol that utilized estrus donor's oocytes and *in vitro* maturation in SOF was superior to the other protocol tested, demonstrating that the association between phase of the estrus cycle and maturation medium is beneficial for oocyte *in vitro* maturation (58% MI). It was demonstrated, in all the protocols tested, that the 24 hours *in vitro* maturation was insufficient for obtaining nuclear configuration of metaphase II.

Key words: Bitches, Maturation; Oocytes; Reproductive Cycle.
Financial support: CAPES
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IN VITRO EMBRYO DEVELOPMENT FROM BOVINE OOCYTES MATURATED IN MEDIUM WITHOUT CONTROLLED GASEOUS ATMOSPHERE

Jasmin.[1,2]; Polisseni, J.[1]; Torres-Júnior, J.R.S.[1]; Sá, W.F.[1]; Camargo, L.S.[1]; Ferreira, A.M.[1]; Folhadella, I.M.[1]; Viana, J.H.M.[1]; Ramos, A.A.[1,3]

[1] Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA Gado de Leite, Juiz de Fora, MG, 36038-330, Brasil. [2] Universidade Federal de Ouro Preto (graduanda), MG, Brasil. [3] Empresa de Pesquisa Agropecuária de Minas gerais – EPAMIG, MG, Brasil. jasminjf@terra.com.br

The success of bovine *in vitro* produced embryos may be affected by the time transport of oocytes to the laboratory once it can influence the maturation process impairing the embryonic development and limiting the embryonic production in commercial scale. The aim of the present study was to evaluate a bovine oocyte maturation protocol without controlled gaseous atmosphere environment. Oocytes (n=552) collected from ovaries of slaughtered cows were selected and randomly distributed in three treatments: control group – oocytes (n=192) maturated in TCM-199 medium with Earle's salts (Gibco Labs., Grand Island, NY) supplemented with 10% of estrus cow serum (ECS) and FSH for 24h at 38.5ºC under atmosphere of 5% CO_2 in air and 95% of humidity; 12h group – oocytes (n=175) matured in tubes in TCM-199 medium with Hanks's salts and buffered with HEPES (Gibco Labs., Grand Island, NY) supplemented with 10% of ECS and FSH, for 12h in water bath at 38.5ºC without controlled gaseous atmosphere. Plus 12h in medium and atmosphere conduction similar to the control group to complete 24h of maturation; and in the 24h group – oocytes (n=185) matured in TCM-199 medium with Hanks's salts and buffered with HEPES (Gibco Labs., Grand Island, NY) supplemented with 10% of ECS and FSH for 24h in water bath at 38.5ºC without controlled gaseous atmosphere. The matured oocytes were fertilized by spermatozoa selected through swim up method. After 22h of fertilization in medium FERT-TALP supplemented with 10 µl/ml of heparin with 2,0 x 106 spermatozoa/ml the presumptive zygotes were co-cultured with granulosa cells in medium CR2aa supplemented with 10% of fetal bovine serum (FBS) in incubator under similar atmospheric conditions of the control group. Cleavage rate was evaluated 72h after fertilization and the blastocysts production rate on days 7 and 8. Hatching rate was evaluated on day 10. The data was evaluated by Chi Square. Cleavage rate in the control group (67.2%) was higher than 12h group ($P<0.05$; 57.1 %) and the 24h group ($P<0.01$; 48.1%) and no difference was observed ($P>0.05$) between the 12h and 24h groups. Blastocysts production on days 7 and 8 and hatching rate on day 10, respectively, were higher in the control group (29.7%, 31.8% e 57.4%) than the 12h group ($P<0.05$; 18.3%, 20.6% e 33.3%) and the 24h group ($P<0.01$; 13.0%, 14.6% e 22.2%), no difference was observed ($P>0.05$) between the 12h and 24h groups. In conclusion, maturation protocol without controlling gaseous atmosphere support embrionary development, however at development rates were lower than conventional maturation *in vitro* protocol.

STIMULUS OF GLUTATHIONE SYNTHESIS IN BOVINE OOCYTES DURING *IN VITRO* MATURATION *

Wolf, A.[1]; Gabaldi, S.H.[2]; Perecin, F.[3]; Garcia, J.M.[3]

[1] Dep. de Rep. An. e Rad. Vet. - FMVZ - UNESP - Botucatu, Brazil. wolfgabaldi@yahoo.com.br
[2] Dep. de Rep. An. - FMVZ - USP - São Paulo, Brazil.; [3] Dep. de Med. Vet. Prev. e Rep. An. - FCAV - UNESP - Jaboticabal, Brazil.

Advanced biotechnologies use IVP embryos as support, wich is performed in three steps of similar importance: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). The goal of this study was to assess the IVM of bovine oocytes in presence of compounds that could stimulate glutathione (GSH) synthesis, in replace to FCS. TCM-199 medium (Gibco BRL Life Technologies™, Carisbad, USA), supplemented with sodium bicarbonate (26mM), pyruvate (0.25mM), amicacine sulphate (75μg/mL), FSH (0.5μg/mL), hCG (100UI/mL) and 17β estradiol (1μg/ml), was used as based-medium (TCM-199b) to evaluate six maturation media, as follows: laboratory control (S = TCM-199b + 10% SFB), BSA control (B = TCM-199b + 0.5% BSA) and adding cysteine (BC = B medium + 825μM of L-cysteine), cysteine-cysteamine (BCC = BC medium + 100μM of cysteamine), cysteine-cysteamine-insuline, transferrin and selenium mix (BCCI = BCC medium + ITS 8μg:8μg:8ng/mL, respectively) and cysteine-ITS (BCI = BC medium + ITS). Oocytes (n=2104) obtained from cow ovaries after slaughter, with compact *cumulus oophorus* and uniform ooplasm, were *in vitro* matured for 24h in incubator at 38.5°C, under 5% CO_2 in air and a humidified atmosphere to verify nuclear maturation rate, by meiosis stage, and cytoplasmatic maturation rate, by migration of cortical granules (CGs) to cytoplasmatic membrane periphery (n=1084) and by GSH intracellular concentration ([GSHi]) (n=1020) (Browne & Armstrong, Methods Molecular Biology, 1998; 108:347-53). Results were analyzed by ANOVA and averages by Duncan Test for nuclear maturation and CGs migration, and by Tukey Test for [GSHi], with $p<0.05$. Nuclear maturation and CGs migration to cytoplasmatic membrane rates did not show significant differences among groups, but the [GSHi] (pmol/oocyte) was higher in BC (4.25), BCC (4.97) and BCCI (4.42) groups in comparison to S (1.83), B (2.63) and BCI (2.23) groups. In conclusion, addition of these thiol components to the IVM medium did not increase nuclear maturation and the CGs migration rates in any groups, but increased [GSHi] in BC, BCC and BCCI groups; the maturation and [GSHi] were independent events during IVM; FCS can be replace with additives in bovine oocyte IVM as evidenced in this study, without impairing nuclear maturation and CGs migration rates.

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INFLUENCE OF GLUTATHIONE SYNTHESIS DURING *IN VITRO* MATURATION OF BOVINE OOCYTES ON EMBRYONIC DEVELOPMENT AND QUALITY *

Wolf, A.[1]; Gabaldi, S.H.[2]; Perecin, F.[3]; Garcia, J.M.[3]

[1] Departamento de Reprodução Animal e Radiologia Veterinária - FMVZ – UNESP, Botucatu-SP, Brazil. [2] Departamento de Reprodução Animal - FMVZ – USP, São Paulo-SP, Brazil. [3] Departamento de Medicina Veterinária Preventiva e Reprodução Animal - FCAV – UNESP, Jaboticabal-SP, Brazil.
wolfgabaldi@yahoo.com.br

Embryonic quality is closely related with antioxidant intracellular concentration accumulated during oocyte maturation. This work aimed to evaluate the influence of glutathione (GSH) synthesis enhancers, added to maturation medium, on the quantity and quality of embryos in vitro cultured (IVC) in two systems. TCM-199 (Gibco BRL Life Technologies™, Carisbad, USA), supplemented with sodium bicarbonate (26mM), pyruvate (0.25mM), amicacine sulphate (75μg/mL), FSH (0.5μg/mL), hCG (100UI/mL) and 17β estradiol (1μg/ml) was used as basis medium (TCM-199b) for the three maturation mediums evaluated: control (S = TCM-199b + 10% FCS); cysteine (BC = TCM-199b + 0.5% BSA + 825μM de L-cysteine) and cysteine-cysteamine (BCC = BC + 100μM of cysteamine). Bovine oocytes (n=3403) from slaughtered cows, showing homogeneous cytoplasm and compact cumulus were in vitro matured (IVM), in the mediums S, BC or BCC, incubated at 38.5ºC, 5% CO_2 in ar and wet atmosphere. After IVM for 24h oocytes were in vitro fertilized in TALP-IVF for 20h, at the same conditions, and in vitro cultivated during seven days in two systems: SOFaa + 2.5% FCS + 0.5% BSA and 5% CO_2 and 20% O_2 atmosphere, or SOFaa + 0.5% BSA and 5% CO_2 and 20% O_2 atmosphere. The in vitro produced (IVP) embryos were evaluated for cleavage and blastocysts production rates, for inner cell mass and trophoblast cells ratio (ICM:TE), assessed by differential staining; and for number of apoptotic cells, assessed by TUNEL staining. Blastocysts produced were classified as superiors when ICM:TE≥1:2 or ICM>24 and as excellents when ICM were equal or higher than ICM average (24) plus standard deviation (12) (ICM+SD=36). Embryo production rates were submitted to ANOVA and means were tested by Duncan's test; embryo's quality were analized by Fisher's test, at $p<0.05$. Cleavage and blastocyts production average rates did not differ among groups, but the IVC system with 5% O_2 produced blastocysts with better ICM:TE ratio, and higher number of ICM cells than the 20% O_2 group (35.09% vs 27.06%, respectively). This group showed again higher frequencies of superior (ICM:TE≥1:2=58.54% vs 31.78% and ICM>24 cells =55.28% vs 34.88%, respectively) and excellent blastocysts (ICM≥36=51.47% vs 26.67, respectively) independently of the maturation medium. However, 5% O_2 system produced Bl with higher number of apoptotic cells in ICM (5% O_2=8.13% vs 20% O_2=6.04%). In conclusion: GSH synthesis stimulus during IVM and the IVC systems did not interfere with cleavege and blastocyst production rates in any of the employed maturation mediums, the culture system with 5% O_2 produced embryos with better ICM:TE ratios, but with higher number of apoptotic cells in ICM; the ability of this IVP embryos to generate pregnancies is still to be tested.

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INFLUENCE OF VOLTAGE-DEPENDENT CALCIUM ION CHANNEL BLOCKERS ON MAMMALIAN OOCYTES IN *IN VIVO* AND *IN VITRO* FERTILIZATION*

Gabaldi, S.H.[1]; Esper, C.R.[2]; Tedesco, A.C.[3]; Wolf, A.[4]; Oliveira, J.A.[5]

[1] Dep. de Reprod. An. - FMVZ-USP, São Paulo, shgabaldi@hotmail.com; [2] Dep. de Med. Vet. Prev. e Reprod. An. - FCAV-UNESP, Jaboticabal; [3] Dep. de Química - FFCLRP-USP, Ribeirão Preto, Brazil; [4] Dep. de Reprod. An.e Radiol. Vet. - FMVZ-UNESP, Botucatu, Brazil; [5] Dep. de Ciências Exatas - FCAV-UNESP, Jaboticabal, Brazil

It is known that the fertilization process is closely related to the calcium ion. There are few reports about the influence of anti-hypertension therapy with *L*-type voltage-dependent calcium ion channel blockers on female fertility. The main goal of this experiment was to check the influence of verapamil, nifedipine and diltiazem on *in vivo* and *in vitro* fertility of hamster oocytes. Hamster females were used for *in vivo* tests and superovulated to *in vitro* experiments. In the Experiment I, oocytes (n=4007) were incubated for 30 min with verapamil (10μM, 100μM, 2mM or 4mM), nifedipine (10μM, 100μM, 500μM or 1mM), diltiazem (10μM, 100μM, 2mM or 4mM), or drug-free medium (control group), and, after this, fertilized with drug-free spermatozoa. *In vitro* fertilization (n=2850) and evaluation of cortical granules exocytosis (n=1157) were evaluated after 6 hour by Hoechst 33342 and fluorescein isothiocyanate-labeled *Lens culinaris* agglutinin stains, by epifluorescent microscopy. In the Experiment II, calcium channel blockers were supplied to females (n=152) twice per day to during 30 days by oral (vo) or intraperitoneal (ip) administration with verapamil (vo: 40mg/kg/d and ip: 5mg/kg/d) nifedipine (vo: 50mg/kg/d and ip: 10mg/kg/d) or diltiazem (vo: 40mg/kg/d and ip: 10mg/kg/d), after this period, they were evaluated through *in vivo* (pregnancy) and *in vitro* fertilization, cortical granules exocytosis (by epifluorescent microscopy) and ultra-structure by transmission electronic microscopy of *in vivo* treated and *in vitro* fertilized oocytes. In the Experiment III, the intracellular calcium concentrations were measured in oocytes (n=800) in the presence of 50μM of calcium ion channel blockers, by Fluo-3AM fluorescent intensity for 720 sec. Results of Experiment I and II were analyzed by Chi-Square Test ($p<0.05$), and, of Experiment III, by ANOVA ($p<0.05$). As results, in the Experiment I, the antagonists presented dose-dependent effects, treated oocytes presented lower *in vitro* fertilization rate when compared to the control group and, in the verapamil and diltiazem groups, cortical granules exocytosis was partial and oocyte activation was not concluded. The Experiment II showed that *in vivo* fertility of treated females did not change among groups; however, *in vitro* fertilization rates in oocytes of calcium antagonists *in vivo* treated females were lower. Nor cortical granules exocytosis neither electronic microscopy detected differences among groups. Progeny of females that received the therapy in the beginning of pregnancy presented lower birth weight and higher mortality rate. In the Experiment III, the antagonists blocked extracellular calcium influx in oocytes (control group = 108.34nM; verapamil = 19.45nM; nifedipine = 32.51nM, diltiazem = 66.36nM). It is concluded that, there were calcium ion channels with sensibility to *L*-type voltage-dependent calcium ion channel blockers in the plasmatic membrane of oocytes. In *in vitro* treatments, these antagonists influenced negatively in oocytes capacity of fertilization, and in verapamil and diltiazem groups, there was not complete activation on fertilized oocytes. Calcium channel blocker therapy did not reduce the fertility in hamster females, but it supplied in the beginning pregnancy caused teratogenic effects.

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ANGIOTENSIN II AND ITS ASSOCIATION WITH INSULIN-LIKE GROWTH FACTOR-I, INSULIN AND FOLLICULAR CELLS ON BOVINE OOCYTE MATURATION AND CONSEQUENT EMBRYO DEVELOPMENT

Stefanello, J.R.; Arruda, J.N.T.; Zamberlam, G.O.; Oliveira, J.F.C.; Gonçalves, P.B.D.

Universidade Federal de Santa Maria, Departamento de Clínica de Grandes Animais Laboratório de Biotecnologia e Reprodução Animal - BioRep, Santa Maria, RS, Brasil. bayard@biorep.ufsm.br

The aim of the present study was to evaluate the association of angiotensin II (Ang II), insulin-like growth factor-I (IGF-I), insulin (Ins) and follicular cells on bovine oocyte maturation on subsequent embryo development. Cumulus-oocyte complexes (COCs) were aspirated from bovine ovaries obtained in an abattoir. The maturation media was TCM-199 with Earle's salts and L-glutamine, supplemented with 25 mM Hepes, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 10% fetal calf serum, 5 µg/ml LH, 0.5 µg/ml FSH, 100 IU/ml penicillin and 50 µg/ml streptomycin. COCs were matured at 39 ºC in an atmosphere of 5 % CO_2 in humidified air, with eight follicular hemisections (follicles of 2-5 mm) in 200 µl with Ang II (10^{-11} M); Ang II + IGF-I (10 ng/ml), Ang II + Ins (5 µg/ml) and two control groups, in the absence of Ang II, IGF-I and Ins, with (control with cells) and without (control without cells) follicular cells for 1 h (1+23 h), 12 h (12+12 h) or 24 h in the presence of follicular cells and its respective treatments + the period to complete 24 h in maturation media. After 24 h of maturation the oocytes were submitted to IVF and subsequent embryo development in SOF media at temperature of 39 ºC and atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in humidified air for nine days. Data were submitted to arcsin transformation, analyzed by ANOVA in a randomized complete block design model, using PROC GLM and treatments compared by contrast statement in SAS program. In 1 + 23 h, cleavage, blastocyst and hatching rates were not different among treatments. In 12 + 12 h, the oocytes matured in the Ang II + IGF-I showed higher blastocyst/total oocytes (43.8±0.6) and hatching/blastocyst (39.3±3.2) rates in comparison with others treatments ($P<0.05$). In 24 h, the oocytes matured in the Ang II + IGF-I group reached similar blastocyst rates those matured in the control group without cells ($P<0.05$). Also, only in this group (Ang II + IGF-I) hatched blastocysts were obtained after 24 h in the presence of follicular cells. In conclusion, insulin and, essentially, IGF-I improve the action of Ang II resulting in a better oocyte cytoplasmic maturation which reflects in high rates of embryo development. Moreover, the oocyte cytoplasmic maturation evaluated through embryo development was improved when the Ang II and IGF-I were present in the maturation media with follicular cells for 12 + 12 hours.

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OXYGEN TENSION IN THE IVM AND IVF OF BOVINE OCYTES: EFFECT ON THE EMBRYONIC DEVELOPMENT AND PREGNANCY RATE

Leivas, F.G.[1,3]; Brum, D.S.[3]; Saliba, W.P.[3]; Alvim, M.T.T.[3]; dos Santos, M.V.[1]; Bernardi, M.L.[2]; Rubin, M.I.B.[1]; Silva, C.A.M.[1]

[1]Embryolab – Laboratory of Animal Embryology – DCGA, HCV, CCR, Federal University of Santa Maria 97.105-900 Santa Maria-RS; [2] Faculty of Agronomy, UFRGS, Porto Alegre, RS.; [3] Nova Índia-Cenatte Embriões, C.P. 570, Uberaba-MG, 38.001-970; fabioleivas@yahoo.com.br

Maintain the O_2 concentration during the *in vitro* maturation (IVM) and fertilization (IVF) process of bovine oocytes in the adequate levels similar to that found in the cow reproductive tract could be an alternative to improve IVP. Thus would decrease the possibility of oxidative stress and would improve embryo quality. In order to evaluate the effect of 5% O_2 concentration in the IVM and IVF process, 11 routines of OPU/IVP were done with 48 cow donors from which 1092 viable oocytes were obtained. The oocytes were homogenously distributed according their quality in groups of 10 to 20 oocytes. The IVM was performed in 100μL of TCM-199 with FSH, LH and 10% of FCS under mineral oil. The oocytes were incubated at 39ºC, for 24h with 5% CO_2 in air (**Group 20% O_2**) or 5% CO_2, 5% O_2, 90% N_2 (**Group 5% O_2**) under saturated humidity. The IVF was performed in 100μL Fert-TALP medium with heparin and PHE using 2 x 10^6 spermatozoa/mL from two *Bos indicus* bulls selected by Percoll gradients. During IVF, the oocytes and spermatozoa were maintained for 18-24h, at 39ºC in satured humidity and gaseous atmosphere with 5% CO_2 in air or 5% CO_2, 5% O_2, 90% N_2. The embryos were incubated in 100μL of SOFaaci (Holm *et al*., 1999), at 39ºC, for 6-8 days with 5% CO_2, 5% O_2 and 90% N_2 in air and saturated humidity. The embryos obtained from five series were evaluated regarding hatching rates at Day 9 (D0=fertilization day). Another 6 series were performed to evaluate the embryos at Day 7 right before they were transferred recipients. The cleavage and blastocyst rates were submitted to ANOVA by GLM procedure (SAS, 1989). The hatching, the quality I blastocyst and the pregnancy rates were compared by Chi-square Test with significance set at 5%. There were no difference in the cleavage (69.6 and 70.4%), embryo development (37.3 and 38.4%) and quality I blastocyst rates in D7 (78.9 and 74.7%) between the groups 5% O_2 and 20% O_2, respectively. The hatching rates at Day 9, considering that the number of matured oocytes was higher in the 5% O_2 group (21.3 vs 10.8). However, the pregnancy rates were similar between IVM and IVF groups: 25.8% (34/132) and 33.6% (49/146) with 5% O_2 and 20% O_2, respectively. The blastocyst rates and quality of the embryos as well as the pregnancy rates were not compromised when IVM and IVF were performed with 5% O_2 concentration.

IN VITRO MATURATION WITH LUNG AIR DURING TRANSPORT OF BOVINE OOCYTES

Mezzalira, A.[1]; Mezzalira, J.C.[1]; Bunn, S.[1]; Werlich, D.E.[1]; Paulini, F.[1]; Wentz, K.C.[1]

Laboratory of Animal Reproduction Prof. Assis Roberto de Bem - Centro de Ciências Agroveterinárias - CAV - Santa Catarina State University - UDESC - Lages SC
mezzalira@cav.udesc.br

The *in vitro* embryo production is a technology already incorporated into the cattle industry, although its methodology is not yet definitive. Many problems like a long distance until the laboratory sometimes hinders the process or demands additional equipment such portable incubators and/or special gas, increasing the costs. The objective of this study was to evaluate the use of lung air as the atmosphere in the maturation process of bovine oocytes. Firstly, the stabilizing capacity of lung air obtained after 15 seconds of apnea was evaluated in an experiment 10 times replicated. After a stabilization period (minimum 2 hours), TCM 199 buffered with bicarbonate, maintained inside a tightly closed bag and inflated with lung air, presented an average pH of 7.54 ± 0.22, while that stabilized in an incubator with 5% CO_2, was 7.43 ± 0.06. In the following experiment, (7 replications), 232 oocytes were submitted to 24 h maturation in Nunc 4-well dishes containing TCM 199 buffered with bicarbonate, previously stabilized and maintained in lung air atmosphere (Treatment 1). On treatment 2, 206 oocytes were maintained in 1.5mL eppendorf tubes in lung air atmosphere, simulating the transport. Oocytes from control group (n=250) were maturated in an incubator with 5% of CO_2. The fertilization and culture procedures were performed in an incubator with 5% CO_2 in air and high humidity, in a similar way for treatments and control groups. It was obtained 77.8% of cleavage and 33.2% of blastocyst rates in treatment 1 (lung air / dishes), 83.7% of cleavage and 34.9% of blastocyst rates in treatment 2 (lung air / eppendorf), and 68.0% of cleavage and 40.3% of blastocyst rates in control group. Data were submitted to ANOVA with 5% significance, in Biostat statistical software. No differences among groups were observed. The use of lung air atmosphere obtained after 15 seconds of apnea, during the maturation of bovine oocytes, allows high rates of embryo development is easy to make and has low costs.

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EVALUATION OF MATURATION BLOCKING AND INVITRO PIG EMBRYOS CULTURE IN PZM3 AND NCSU23 MEDIA

Marques, M.G.; Nascimento, A.B.; Oliveira, V.P.; Coutinho, A.R.S.; Caetano, H.V.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil – mgroke@usp.br

The aim of this study was to evaluate PZM3 and NCSU23 media for pig embryos culture. Oocytes from slaughterhouse ovaries were randomly allotted in 2 groups: in vitro maturation for 44 hours or blocking for 10 hours with 5µM cyclohexemide and maturation for 40 hours. The oocytes were matured in TCM199 medium supplemented with 3.05mM glucose, 0.91mM sodium pyruvate, 10% pig folicular fluid, 0.57mM cystein, 10ng EGF/ml, 10IU eCG/ml and 10IU hCG/ml for 22 hours and subsequent incubation for 22 or 18 hours (control and blocking, respectively) in medium without hormones. For *in vitro* fertilization, fresh semen was capacitated during fertilization. After maturation, cumulus cells were removed and oocytes incubated with sperm for 6 hours. The presumably zygotes were distributed in PZM3 (groups 1A and 2A) and NCSU23 (groups 1B and 2B) culture media. Comparing cleavage rates on third day of culture, PZM3 (group 2A – 68.4%) showed better result than NCSU23 (group 2B – 44.4%) in groups with blocking, but in groups without blocking there were no differences between PZM3 (group 1A – 50.6%) and NCSU23 (group 1B – 52.5%). On 7th day of culture, group 1A (13.4%) had better blastocyst rate than group 1B (5.6%) and no difference were observed between groups 2A (6.3%) and 2B (9.8%). In conclusion, maturation blockage did not improve blastocyst rates on IVF pig embryos and PZM3 was more efficient on embryo culture than NCSU23.

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EVALUATION OF THE INFLUENCE OF GH, IGF-1 AND INSULIN ON BOVINE OOCYTE *IN VITRO* MATURATION AND EMBRYO PRODUCTION

Ponchirolli, C.B.; Freitas, C.P.; Landim-Alvarenga, F.C.

Departamento de Reprodução Animal e Radiologia Veterinária- FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. carlaponchi@yahoo.com.br

In vitro embryo development is potentially affected by several factors, including the quality of *in vitro* maturation process. GH (growth hormone), IGF-1 (insulin like growth factor) and insulin could potentially improve oocyte maturation rates when added to the *in vitro* maturation medium. The objective of the present study was to evaluate the influence of the addition of GH, IGF-1 and insulin to bovine oocyte *in vitro* maturation medium (IVM), on blastocyst production. Ocytes were obtained from 2 and 8 mm follicles from Bovine slaughterhouse ovaries. After selection oocytes were matures in 5 different maturation media. Control group: medium TCM 199 supplemented with 10% bovine foetal serum (BFS), 2,2 mg/ml sodium pyruvate, 1 mg/ml estradiol 17ß, 50 µg/ml LH, 5 µg/ml FSH and 75 µg/ml gentamicin (control medium); GH group: control medium + 100 ng/ml GH; IGF-1 medium: control medium + 10 ng/ml IGF-1; insulin group: control medium +1µg/ml insulin; GH+IGF-1+Insulin group: control medium + 100 ng/ml GH, 10 ng/ml IGF-1+ 1µg/ml insulin. Culture was performed at 38º C, in a humidified atmosphere of 5% CO_2 in air for 22-24h: After maturation, oocytes were fertilized in Fert Talp for 12h, under the same conditions. Presumptive zygotes were placed in 90 µl drops and cultured in presence of granulosa cells (co-culture) in HTF medium (Human Tubal Fluid, HTF®, Irvane) and BME (Basal Medium Eagle, BME®, Sigma), on 1:1 rate, supplemented with 10% BFS, 0,01% myo-inusitol and 75 µg/ml gentamicin; For statistical analysis ANOVA was used. There were no statistical differences between groups. Control:154 blastocysts/ 532 oocytes -29%; GH: 151/539–28%; IGF-1: 154/535 –28%; insulin: 141/539–26%; GH+IGF-1+insulin: 1445/527- 27%. The present study showed that the addition of GH, IGF-1 and insulin or all of them to *in vitro* oocyte maturation medium didn't improve blastocyst production. These unespected results may be due to presence of BFS in maturation medium. There is a variety of growth factors present in BFS that could mask the role of the stimulatory factors added to the medium.

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EFFECTS OF ANTIOXIDANTS AND *CUMULUS* CELLS ON *IN VITRO* MATURATION OF BOVINE OOCYTES

Gotardi, F.P.[1]; Barretto, L.S.S.[2]; Gonçalves, F.S.[2]; Perri, S.H.V.[1]; Mingoti, G.Z.[1]

[1] Veterinary Medicine, UNESP, Araçatuba-SP, 16050-680, Brazil. [2] Faculty of Agrarian and Veterinary Sciences, UNESP, Jaboticabal-SP, 14884-900, Brazil. gmingoti@fmva.unesp.br.
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Glutathione (GSH) plays an important role in protecting the cell from oxidative damage. It has been suggested that *cumulus* cells during *in vitro* maturation (IVM) play an important role in oocyte GSH synthesis. The present study was carried out to evaluate the role of *cumulus* cells during IVM of bovine oocytes in the presence of the antioxidant cysteamine. Ovaries were collected at a slaughterhouse and 2-6mm follicles were aspirated to obtain oocytes. Oocytes (n=555) were matured *in vitro* at 38.5ºC under a 5% CO_2 in air environment, in a 0.6% BSA supplemented TCM-199 media with 0.5µg FSH, 100 IU hCG, and 1µg estradiol/mL (*Control*) with either 50 µM cysteamine (*Cist*). Three groups were performed: intact *cumulus*-oocyte-complexes (COC), denuded oocytes (DO), and denuded oocytes plus the removed *cumulus* cells (DO+CC). After 24 hours maturation, the oocytes were stained with Hoechst 33342 to evaluate nuclear maturation, and were classified as germinal vesicle (GV - immature), metaphase I (incomplete maturation) or metaphase II (MII - mature). Data were evaluated through the ANOVA followed by Duncan test ($P<0.05$). After 24 hours of maturation, we observed maturation (MII) in 69.81%^{a} (COC), 62.86%^{ab} (DO) and 57.26%^{b} (DO+CC) of oocyte matured in *Contr* medium. For oocytes matured in *Cist* medium, we observed MII in 86.32%^{a} (COC), 78.39%^{ab} (DO) and 67.39%^{b} (DO+CC). Data demonstrated a beneficial effect of *cumulus* cells during IVM, and also demonstrated that coupling between *cumulus* cells and oocytes was advantageous for nuclear maturation. Additionally, we also performed a contrast statement analysis (PROC GLM) to evaluate the effect of cysteamine on IVM, and we found a higher percentage of oocytes reaching MII when IVM occurred in presence of cysteamine ($P<0.05$). These data demonstrated an advantageous effect of cysteamine on nuclear maturation.

EFFECTS OF NUCLEAR MATURATION INHIBITORS AND ANTIOXIDANTS ON THE *IN VITRO* MATURATION OF BOVINE OOCYTES

Barretto, L.S.S.[1]; Gonçalves, F.S.[1]; Perri, S.H.V.[2]; Mingoti, G.Z.[2]

[1] DMVPRA, FCAV-UNESP, Jaboticabal-SP, Brasil. [2] DAPSA, FMVA-UNESP, Araçatuba-SP, Brasil.
leticiabarretto@ig.com.br

The nuclear maturation inhibitors butirolactone I and roscovitine maintain bovine oocytes at the germinal vesicle stage in a reversible way. Antioxidants (cysteamine and β-mercaptoethanol) protect the cell from oxidative damage into the culture medium. We objectify to evaluate the effect of butirolactone I, roscovitine, cysteamine and β-mercaptoethanol in the *in vitro* nuclear maturation of bovine oocytes. Oocytes (n=489) were matured at 38,7°C under a 5% CO_2 in air environment, in a 0,6% BSA supplemented TCM-199 medium with 0,5 μg FSH, 100 IU hCG and 1 μg estradiol/mL (control group - C) with either 50 μM cysteamine (CC group) or 50 μM β-mercaptoethanol (CM group). The standard group of the laboratory (S group) was supplemented with 10% FCS as protein source. The oocytes in which groups with butirolactone I and roscovitine were pre incubated for 24 hours in a 0,3% BSA supplemented TCM-199 medium with antibiotic and pyruvate, and then transferred to the control group medium for maturation as according to the groups: 100 μM butirolactone I (B), 100 μM butirolactone I + 50 μM cysteamine (BC), 100 μM butirolactone I + 50 μM β-mercaptoethanol (BM), 25 μM roscovitine (R), 25 μM roscovitine + 50 μM cysteamine (RC) and 25 μM roscovitine + 50 μM β-mercaptoethanol (RM). After 24 hours of maturation, the oocytes were stained with Hoechst 33342 to evaluate nuclear maturation stage. Data were analysed using ANOVA ($P<0.05$). After 24 hours maturation, the oocytes came in metaphase II stage in 66.32% (S), 65.85% (C), 66.67% (CC), 64.61% (CM), 70.37% (B), 56.22% (BC), 55% (BM), 57.90% (R), 60.80% (RC) and 59.20% (RM), with no significant differences ($P>0,05$) among the groups. We have verified the reversibly of meiotic resumption after the removal of butirolactone I and roscovitine medium.

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INFLUENCE OF GLUTATHIONE PRECURSORS DURING *IN VITRO* MATURATION (IVM) ON EMBRYONIC DEVELOPMENT OF BOVINE OOCYTES

Saraiva, N.Z.[1]; Tetzner, T.A.D.[1]; Borges, J.C.[1]; Wolf, A.[1]; Ferreira, C.R.[1]; Perecin, F.[1]; Méo, S.C.[2]; Vantini, R.[1]; Garcia, J. M.[1]

[1]DMVPRA FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil, [2]CPDGRA-IZ, APTA/SAA, Nova Odessa-SP, 13460-000, Brazil. naiaravet@yahoo.com.br

Glutathione (GSH) is composed by the aminoacids glutamate, glycine, and cysteine and its main role is to protect the cell membrane against free radicals. The aim of this study was to evaluate the effects of GSH precursors, in maturation medium, on early embryonic development in bovine. Oocytes obtained from ovaries of slaughtered cows presenting homogeneous cytoplasm and compact *cumulus* were selected, transferred in groups of 25 to 100μL drops, under mineral oil, with specific maturation medium for each treatment and cultured at 38.5ºC and 5% CO_2 in air, for 24 hours. The standard maturation medium was sodium bicarbonate-buffered (2.2mg/mL) TCM 199 with Earle's salts (GibcoBRL), supplemented with 1μg/mL FSH, 100UI/mL hCG, 1μg/mL estradiol, 0.25mM sodium pyruvate, and 75μg/mL amicacin. For each one of the 5 treatments, the standard medium was supplemented with: control group (C) – 10% FCS (n=221), BSA control group (B) – 0.5% BSA (n=235), BSA+Cysteine group (BC1) – medium B with 825μM L-cysteine (n=227), BSA+Cysteamine group (BC2) – medium B with 100μM cysteamine (n=245) and BSA+Cysteine+Cysteamine group (BCC) – medium B with 825μM L-cysteine and 100μM cysteamine (n=231). Twenty-four hours after IVM, the oocytes were fertilized in TALP-IVF medium for 20 hours and cultured in SOFaa supplemented with 2.5% FCS and 0.5% BSA. Cleavage and blastocyst production rates were assessed, respectively, 48 hours and 7 days after IVF. Four replicates were done, and the results were analyzed by ANOVA and the means were tested by Duncan's test ($P<0.05$). For cleavage rates, BC1 (73.98%) was superior to BC2 (63.28%) and BCC (60.60%) groups, and similar to C (64.97%) and B (71.79%) groups. The B group was superior to BCC group for cleavage. However, for blastocyst production, the BC2 group (59.95%) was superior to all the other groups (C = 49.86%; BCC = 44.79%; B = 43.68%; BC1 = 37.71%). C group produced more blastocysts than BC1 group, and the BCC and B groups were similar to C and BC1 groups. Although the BC1 group has presented higher cleavage rates, blastocyst production rates were higher for the BC2 group. These results are supported by data observed in ovine species (DE MATOS et al., Theriogenology, v.57, p.1443-51, 2002), in which the treatment with cysteamine alone favored blastocyst development. It was not achieved the expected increase in blastocyst production for groups supplemented with cysteine (BC1 and BCC). One hypothesis is that the excess of thiols in the medium could disrupt cell balance and interfere on embryonic development.

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MITOCHONDRIA DISTRIBUTION IN BOVINE OOCYTES MATURED *IN VITRO* AFTER MEIOSIS BLOCK

Pires, P.R.L.[1,3]; Adona, P.R.[2,3]; Quetglas, M.D.[3]; Leal, C.L.V.[3]

[1]UNIARARAS, Araras-SP, [2]Setor de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, [3]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, paulo_adona@yahoo.com.br

The distribution of mitochondria within the cytoplasm of the bovine oocyte is important for maturation, fertilization and embryo development. One of the main roles of mitochondria is to provide energy for cell maintenance and division. The present study aimed to assess the effect of meiosis block on the distribution of mitochondria in bovine oocytes after *in vitro* maturation (IVM). Ovaries were collected at a slaughterhouse and 2-6mm follicles were aspirated to obtain oocytes. Meiosis progression of oocytes was blocked for 24h in three different treatments, in four replicates: a) BL10 (n=127) - 10μM butyrolactone I (BL); b) BL100 (n=118) - 100μM BL and 3mg/ml BSA and c) RB (n=119) – 6.25μM BL and 12.5μM roscovitine (R). All treatments were diluted in TCM199. After meiosis block, oocytes were *in vitro* matured for 24h (TCM199 with 10% FCS, 0.5μg/ml FSH and 5.0μg/ml LH. A control group (24h control, n=59) was submitted to IVM only. Oocytes were assessed after block and IVM. A group of oocytes was yet assessed immediately after aspiration (0h control, n=63). To evaluate mitochondria distribution oocytes were denuded and stained with 0.5μM mito-tracker (red) in PBS with 0.1% PVA for 20 min; stained with 10μg/ml hoechst 33342 in PBS with 0.1% PVA for 10 min; washed three times in PBS with 0.1% PVA and observed under an epifluorescence microscope. Data were analyzed by the GLM - PROC GENMOD method of SAS. In immature oocytes, 92 to 100% of them were in germinal vesicle, irrespective of treatment ($P>0.05$). Mitochondria were distributed mainly in the peripheral area of the cytoplasm, at 0h control (100%) and after meiosis block for 24h for BL10 (82%), BL100 (87%) and RB (74%), $P>0.05$. After IVM (96 to 100% of oocytes were in metaphase II, $P>0.05$), however, mitochondria were evenly distributed throughout the cytoplasm, at 24h control group (83%) and blocked prior IVM group for BL10 (96%), BL100 (98%) and RB (100%), and in these, migration was superior to control ($P<0.05$). It is concluded that meiosis block also inhibits mitochondria migration, but after IVM this migration can be stimulated. Financial support Fapesp 03/01479-6 and 04/14166-9, and Capes.

EFFECT OF MEIOSIS BLOCK ON CORTICAL GRANULE MIGRATION IN BOVINE OOCYTES MATURED *IN VITRO*

Adona, P.R.[1,2]; Pires, P.R.L.[2,3]; Quetglas, M.D.[2]; Leal, C.L.V.[2]

[1]Laboratório de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, [2]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, [3]UNIARARAS, Araras-SP, paulo_adona@yahoo.com.br

Cortical granules (CG) are organelles produced by the Golgi apparatus and are present only in female gametes. Its main function is to modify the zona pelucida and impair polyspermy. CG are formed in growing oocytes, but the reorganization of its distribution occurs during maturation. The aim of this work was to assess the effect of meiosis block in CG migration in bovine oocytes submitted to posterior *in vitro* maturation (IVM). Ovaries were collected at a slaughterhouse and 2-6mm follicles were aspirated to obtain oocytes. Meiosis block in oocytes was induced by 24h culture in three different treatments, in four replicates: a) BL10 (n=116) - 10μM butyrolactone I (BL); b) BL100 (n=116) - 100μM BL and 3mg/ml BSA and c) RB (n=115) – 6.25μM BL and 12.5μM roscovitine (R). All treatments were diluted in TCM199. After meiosis block, oocytes were *in vitro* matured for 24h (TCM199 with 10% FCS, 0.5μg/ml FSH and 5.0μg/ml LH. A 24h control group (n=59) was submitted to IVM only and a 0h control group (n=56) was evaluated immediately after aspiration. Oocytes were assessed after block and IVM. Oocytes were denuded and the zona pelucida removed with 0.5% pronase in PBS for about 5 min and fixed in 3.7% de paraformaldehyde in PBS with 0.1% PVA for 30 min. Next, oocytes were incubated in SB (PBS with 0.1% BSA, 0.75% glycine and 0.2% sodium azide) for 2h; then with 0.1% Triton X-100 in SB SB for 5min; washed three times in SB; stained with 1μg/ml *Lens culinaris* FITC conjugated plus 10μg/m propidium iodide in SB for 15min; washed again three times in SB and observed under an epifluoresence microscope. Data were analyzed by the GLM - PROC GENMOD method of SAS. In 100% of non matured oocytes (96-100% in germinal vesicle stage, $P>0.05$), after aspiration (0h control) and in blocked oocytes prior to IVM, CG were distributed throughout the cytoplasm ($P>0.05$). However, after IVM, 96 to 98% ($P>0.05$) of the treated and 24h control oocytes showed their CG in the periphery of the cytoplasm underneath the plasma membrane. It can be concluded that meiosis block also impairs CG migration, but after removal of the inhibitory stimulus, CG migrate normally after IVM.

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EFFECT OF DIFFERENT BUTYROLACTONE I CONCENTRATIONS AND PRESENCE OF BSA ON MEIOSIS BLOCK IN BOVINE OOCYTES

Adona, P.R.[1,3]; de Bem, T.H.C.[2,3]; Leal, C.L.V.[3]

[1]Setor de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, [2]UNIARARAS, Araras-SP , [3]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, paulo_adona@yahoo.com.br

Several studies have been developed aiming to improve the competence of *in vitro* matured oocytes. A possible alternative is the meiosis block of oocytes before submitting them to *in vitro* maturation (IVM). Specific maturation promoting factor (MPF) inhibitors, such as butyrolactone I (BL) have been used for this purpose. The aim of the present study was to assess the effect of different BL concentrations in the presence or not of BSA on the meiosis block in bovine oocytes. Slaughterhouse ovaries were collected and oocytes aspirated from 2-6mm follicles. In experiment 1 BL was diluted in TCM199 at concentrations of 0 (control, n=73), 10 (n=78), 15 (n=72), 20 (n=76) and 25μM (n=76). In experiment 2, oocytes were cultured with 0 (control, n=77), 25 (n=83), 50 (n=77) and 100μM BL (n=78) in TCM199 in the presence of 3mg/mL BSA. In both experiments, after the block period (24h) the oocytes were denuded, fixed in ethanol/acetic acid (3:1) and stained with 1% lacmoid to determine the stage of meiosis. Data were analyzed using the GLM - PROC GENMOD method of SAS. Experiments were done in three replicates. In experiment 1, 95 to 97% ($P>0.05$) of the ocytes remained in germinal vesicle (VG) in BL treated groups, while in the control (no BL) only 14% of the oocytes ($P<0.05$) remained at this stage, suggesting that in the absence of BSA low BL concentrations are able to block meiosis. In experiment 2, as BL concentration increased in the presence of BSA, so did the meiosis block rate (65, 84 and 98% VG for 25, 50 and 100μM BL, respectively, $P<0.05$). In controls, no oocytes remained at this stage. These data show that in the presence of BSA, higher concentrations of BL are necessary to block meiosis. In conclusion, the efficiency of meiosis block is related to the concentration of the inhibitor and the supplementation of the inhibition medium.

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GENE EXPRESSION IN BOVINE OOCYTES AND CUMULUS CELLS BEFORE AND AFTER IN VITRO MATURATION WITH AND WITHOUT LH – PRELIMINARY RESULTS

Mundim, T.C.D.; Franco, M.M.; Brandão, D.O.; Ávila, F.F.; Melo, E.O.; Rumpf, R.

Laboratório de Reprodução Animal, EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, DF, Brasil. Avenida W/5 Norte Final, PqEB, Prédio Biotecnologia, Sala 7B, 70770-900.
mfranco@cenargen.embrapa.br

Many new biotechniques applied to animal reproduction have been developed to optimize animal production. In vitro conditions for maturation, fertilization and culture have been studied, stimulating the search for new protocols that improve techniques and reduce costs. Apart from the parameters currently used, such as cytoplasm uniformity, number and expansion of cumulus cells layers, molecular biology, trying to identify molecular markers and processes involved in differential gene expression is a potential tool to monitor and evaluate in vitro embryo production (IVP) systems. In this context, the objective of the present study was to detect the expression of IGF2, IGF2r, GRB10 and GDF9 genes, which are involved in oocyte maturation. Immature and mature cumulus-oocyte complexes obtained from slaughterhouse ovaries were used. Follicles were aspirated and oocytes selected and matured with LH (Control) and without LH, as routinely performed in the Laboratório de Reprodução Animal da Embrapa Recursos Genéticos e Biotecnologia. Total RNA was isolated from immature and matured oocytes with and without LH and from their respective cumulus cells and RT-PCR reaction was performed, and the products were visualized in agarose gel stained with ethidium bromide. There was a clear differential expression for the IGF2 gene between the cumulus cells before and after maturation and for the GRB10 gene which was more intensively expressed in oocytes matured without LH. Although these are preliminar results, this work demonstrates the potential use of molecular markers to evaluate and monitor in vitro embryo production (IVP) systems.

MICROFILAMENT DISTRIBUTION DURING *IN VITRO* MATURATION OF BOVINE OOCYTES AFTER MEIOSIS BLOCK

Adona, P.R.[1,2]; Valério, J.[2]; Leal, C.L.V.[2]

[1]Setor de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, [2]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, paulo_adona@yahoo.com.br

Microfilaments are part of the cellular cytoskeleton and are formed by actin filaments. During maturation they are responsible for altering the position of cortical granules. The present study aimed to assess microfilament distribution during *in vitro* maturation (IVM) of oocytes previously submitted to meiosis block. Ovaries were collected at a slaughterhouse and 2-6mm follicles were aspirated to obtain oocytes. Meiosis progression of oocytes was blocked for 24h in TCM199 medium with three different treatments: a) BL10 - 10μM butyrolactone I (BL); b) BL100 - 100μM BL and 3mg/ml BSA and c) RB – 6.25μM BL and 12.5μM roscovitine (R). After meiosis block, oocytes were *in vitro* matured for 24h (TCM199 with 10% FCS, 0.5μg/ml FSH and 5.0μg/ml LH) and assessed at 0, 6, 12, 18 and 24h post-block IVM. A control group was submitted to IVM only and assessed at the same times. For the evaluation, oocytes were denuded and fixed in 3.7% paraformaldehyde and 0.6% Triton X-100 in PP (0.1% PVA in PBS) for 30 min; stained with 1μg/ml FITC conjugated phalloidin and 10μg/ml propidium iodide for 30 min; washed three times in PP and observed under an epifluorescence microscope. Data (stages of meiosis) were analyzed by the GLM - PROC GENMOD method of SAS. Microfilament distribution had descriptive evaluation. At the beginning of culture (0h) the oocytes were in germinal vesicle stage (92-100% VG, $P>0.05$). After 6h IVM, the proportion of VG oocytes in the treated group (55-66%, $P>0.05$) was reduced compared to the control (83% VG, $P<0.05$), indicating that treated oocytes resumed meiosis earlier. After a 12h IVM, most oocytes (87-96%, $P>0.05$) in all groups were at intermediate stages of meiosis (metaphase I to telophase I). At 18 and 24h, most oocytes had already matured to metaphase II (68-72% and 90-98%, respectively, $P>0.05$). Microfilaments were observed throughout the cytoplasm of oocytes in all groups and times studied. At 18 and 24h IVM, a stronger staining was observed around the area where the polar body was released. No differences or alterations were observed in microfilament distribution in the cytoplasm of any of the oocytes analyzed. It can be concluded that meiosis block under the conditions studied induces acceleration in meiosis resumption, but the speed of meiosis progession tends to normalize along IVM. Microfilaments were not adversely affected by the treatments.

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MEIOSIS BLOCK OF BOVINE OOCYTES AND MICROTUBULE ORGANIZATION DURING *IN VITRO* MATURATION

Pires, P.R.L.[1,3]; Adona, P.R.[2,3]; Leal, C.L.V.[3]

[1]UNIARARAS, Araras-SP, [2]Setor de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ, [3]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, paulo_adona@yahoo.com.br

Microtubules are cytoplasmic structures of the cellular cytoskeleton. They are the main components of the meiosis spindle, providing structure for cell division, besides participating in the transport and organization of organelles and particles within the cytoplasm. The aim of this study was to assess the microtubule organization during the *in vitro* maturation (IVM) of bovine oocytes submitted to meiosis block. Ovaries were collected at a slaughterhouse and 2-6mm follicles were aspirated to obtain oocytes. Meiosis progression of oocytes was blocked for 24h in TCM199 medium with three different treatments: a) BL10 - 10μM butyrolactone I (BL); b) BL100 - 100μM BL and 3mg/ml BSA and c) RB – 6.25μM BL and 12.5μM roscovitine (R). After meiosis block, oocytes were *in vitro* matured for 24h (TCM199 with 10% FCS, 0.5μg/ml FSH and 5.0μg/ml LH) and assessed at 0, 6, 12, 18 and 24h post-block IVM. A control group was submitted to IVM only and assessed at the same times. For the evaluation, oocytes were denuded and fixed in 3.7% paraformaldehyde and 0.6% Triton X-100 in PP (0.1% PVA in PBS) for 30 min; blocked in 3% goat serum in PBS for 45 min; stained with anti—αtubulin FITC conjugated (1:100) in PP for 1h; stained with 10μg/ml propidium iodide for 15 min and observed under an epifluorescence microscope. Data (stages of meiosis) were analyzed by the GLM - PROC GENMOD method of SAS. Microtubules distribution had descriptive evaluation. At 0h IVM, most oocytes (87-100%, $P>0.05$) were in germinal vesicle stage (GV). After 6h, however, treated groups had less oocytes in GV (~35%, $P>0,05$) than controls (64%, $P<0.05$), indicating an earlier meiosis resumption. At 12h, the majority of the oocytes (73-78%, $P>0.05$) were in intermediate meiosis stages. At 18 and 24h IVM, most oocytes had reached metaphase II (72-82% and 90-93% MII, respectively, $P>0.05$). Microtubules showed positive staining throughout the cytoplasm in all oocytes evaluated. However, a more intense staining was observed in the spindle at 6h IVM (more frequent in treated oocytes), which became even brighter 12 and 18h. At 24h IVM, microtubule staining was less intense. No alterations in the spindle were observed regarding organization and formation of the metaphase plate in the different groups and times studied. In conclusion, meiosis block with the treatments used accelerates initial steps of meiosis, which regain normal speed along IVM. Treatments did not negatively affect microtubules.

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MEIOSIS BLOCK WITH BUTYROLACTONE I DOES NOT INFLUENCE MITOCHONDRIAL DNA CONTENT IN BOVINE OOCYTES *IN VITRO*

Biase, F.H.[1]; Adona, P.R.[1,2]; Miranda, M.S.[1]; Leal, C.L.V.[1]; Meirelles, F.V.[1]

[1]Departamento de Ciências Básicas – FZEA/USP, Pirassununga-SP, [2]Setor de Reprodução Animal – LMGA/CCTA/UENF, Campos dos Goytacazes-RJ

Mitochondria are bioenergetic centrals with a essential function which defines oocyte functional competence. They are specialized organelles responsible for the production of the major part of cellular energy in the form of ATP. During mitosis mitochondria are randomly distributed among daughter cells and along oogenesis there is a substantial increase in their number, with an expressive variation among oocytes in the same developmental stage. There is a similar correlation among developmental potential, ATP content and mitochondrial function in bovine oocytes and embryos. The aim of this work was to verify the variation in mitochondrial DNA (mtDNA) amount between bovine oocytes cultured *in vitro* in the presence or absence of butyrolactone I (BL). Ovaries were collected in slaughterhouse and 2-8 mm follicles were aspirated to obtain oocytes. Oocytes were used to assess the quantity of mtDNA after being submitted to 3 treatments: T1 – blocked for 24h in TCM199 with 10μM de BL, T2 – blocked for 24h and then *in vitro* matured after the blocked for another 24h (TCM-199 with 10% FCS, 0.5μ/mL FSH, 5.0μg/mL LH and 10μg/mL gentamycin) and T3 – only *in vitro* matured (control) without prior blockage. A group of oocytes was collected for evaluation immediately after aspiration (0h). All oocytes were denuded and individually frozen in 1μL PBS at -80ºC. Twelve oocytes from each group were individually submitted to PCR. mtDNA amplification was performed by real time PCR using specific primers and the fluorescence detection was made using SYBRGreen. The Cycle Threshold (Ct) data from each amplification was considered as a variable to compare the amounts of mtDNA in each oocyte. Statsitical analysis was performed by ANOVA for non parametric data. Means of Cts in groups 0h, T1, T2 and T3 were 26.33, 26.26, 26.73 and 24.96, respectively. There was no difference ($p>0.05$) among Cts of each group analyzed. The results suggest that *in vitro* cultured bovine oocytes in the presence of BL does not interfere with their amount of mtDNA.

ETHIDIUM BROMIDE IMPACT ON *IN VITRO* CYTOPLASMIC AND NUCLEAR MATURATION OF BOVINE OOCYTES

Ferreira, C.R.[1]; Meirelles, F.V.[2]; Perecin, F.[1]; Méo, S.C.[3]; Tetzner, T.A.D.[1]; Saraiva, N.Z.[1]; Yamazaki, W.[1]; Vantini, R.[1]; Garcia, J.M.[1]

[1]DMVPRA FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil, [2]FZEA-USP, ZAB, Pirassununga-SP, Brazil, [3]CPDGRA – IZ, APTA/SAA, Nova Odessa-SP, 13460-000, Brazil.
chrisferreira13@yahoo.com.br

Mitochondrial DNA (mtDNA) inheritance has been of great interest in the research area of reproductive biotechnologies, such as nuclear transfer, ICSI and cytoplasmic transfer. Control of mtDNA inheritance may be useful to allow selection of the mtDNA haplotype of interest to be transmitted to the progeny. The aim of this work was to evaluate the effect of Ethidium Bromide (EB) on *in vitro* nuclear and cytoplasmic maturation of bovine oocytes. EB can be tested in biological models involving the strategy of impairing one mtDNA haplotype replication in order to allow the second one to multiply. Oocytes obtained from cows ovaries collected in slaughterhouse (n=803) and presenting compact *cumulus* and uniform cytoplasm were divided in 1 control and 7 experimental groups (mean of 33 oocytes/group, 3 repetitions) treated with increasing concentrations of EB (Sigma, St. Louis, USA): 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 7.0µg EB/mL. IVM was performed in TCM-199 medium supplemented with 10% FCS, FSH (1µg/mL), hCG (50µg/mL), estradiol (1µg/mL), sodium pyruvate (0.20mM), amicacin (83.4µg/mL), and 50ng/mL of uridine 5´-triphosphate under 5% CO_2 in air at 38.5°C. Each group was placed in a separated well of a 24-well plate. After 24h IVM, oocytes were stained according to Cherr *et al*. (Journal of Experimental Zoology 246, 81-93, 1988) and evaluated under epifluorescence microscopy for cortical granule (CG) migration (fluorescein isothiocyanate, wavelengths 490/520nm), indicative of cytoplasmic maturation and for progression to metaphase II (Hoechst 33342, wavelenghts 355/465nm), indicative of nuclear maturation. Results were analyzed by Duncan´s multiple range test using SAS. Impairment of CG migration was significant ($P<0.05$) in concentrations superior to 2.5µg/mL EB, as showed by increased absent (0 and 9.87% in control and treatment, respectively) and decreased complete CG migration (50.75 and 13.58% in control and treatment, respectively). Partial CG migration was significantly different from the control group at 2.5, 4.5 and 7µg/mL EB (76.34, 77.96 and 76.86%, respectively). A significant harmful effect on CG migration was not observed at EB concentrations up to 1.5µg/mL EB in IVM bovine oocytes. Metaphase II presence, indicative of nuclear maturation, was proportionally impaired with increasing EB concentrations, with significant impairment with 2.5µg/mL EB and higher concentrations (58.87 and 22.22% in control and in 2.5µg/mL EB, respectively, $P<0.0001$). During oocyte maturation, granulosa cells present high metabolism and supply energy to the oocyte. EB treatment may damage these cells metabolism, reflecting in oocyte nuclear and cytoplasmic maturation. According to these results, EB concentrations up to 1.5µg/mL during 24 h IVM didn't disturb significantly cytoplasmic and nuclear maturation. If this concentration range will be enough to guarantee receptor embryo mitochondrial haplotype replication impairment after cytoplasmic transfer, is still to be tested.

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DYNAMICS OF THE CONCENTRATION OF INTRACELLULAR GLUTATHIONE *IN VITRO* MATURED BOVINE OOCYTES IN MEDIUM SUPPLEMENTED WITH PRECURSORS OF GLUTATHIONE

Tetzner, T.A.D.[1]; Saraiva, N.Z.[1]; Wolf, A.[1]; Ferreira, C.R.[1]; Perecin, F.[1]; Borges, J.C.[1]; Méo, S.C.[2]; Vantini, R.[1]; Garcia, J.M.[1]

[1]DMVPRA FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil, [2]CPDGRA-IZ, APTA/SAA, New Odessa-SP, 13460-000, Brazil. tatiane_tetzner@yahoo.com.br

The glutathione (GSH) is a basic tripeptídeo for the protection of the cell against the oxidative stress and it participates in the processes of oocyte maturation. Its synthesis in oocytes is indicative reliable of citoplasmic maturation. The objective of that experiment was to evaluate the intracellular concentration of GSH in immature oocytes (0 hour), and during the process of IVM (8, 16 and 24 h), in medium containing thiol components or not. Oocytes (n=1050) obtained from ovaries of slaughterhouse cows and presenting cytoplasm homogeneous and compact *cumulus*, were distributed in seven experimental groups in five repetitions. Immature oocytes were collected (0h) and the remaining was distributed in two experimental groups, control (CON): TCM-199 sodium bicarbonate-buffered, supplemented with FCS (10%) and hormones; and BSA + Cysteine +Cysteamine (BCC): TCM-199 sodium bicarbonate-buffered, supplemented with BSA (0.5%) and hormones, added of L-cysteine (825µM) and cysteamine (100µM), for culture in humid greenhouse (to) at 38.5ºC and 5% of CO_2 in air, during 8 (8HCON and 8HBCC), 16 (16HCON and 16HBCC) or 24 hours (24HCON and 24HBCC). The concentration of GSH was measured by the protocol of Browne and Armstrong (Methods in molecular biology, v. 108, p.347-353, 1998) with adaptations. For each time of collection, 30 oocytes CON and BCC had the cells of the *cumulus* removed with hyaluronidase (0.2%) and deposited in microtubo, containing 100 µL of phosphoric acid extraction solution (SEAM). The content was homogenized with needle (29G ½) for the lise, and stocked to –20ºC until the reading. The samples were centrifuged (1500RPM) and the sobrenadante (100 µL) transferred for a test tube, containing 2mL of glutathione lid solution (TG), to which 100 µL of the reagent was added o-phthaldialdehyde (OPT). The reading was accomplished, after incubation for 15min in the darkness, in espectrofluorímetro (Hitachi-F-2500, Tokyo, Japan) fitting for emission in 420 nm and excitement in 350nm. The results were analyzed by ANOVA and the means by Tukey's test. There was not statistical difference when the treatments CON and BCC were compared in the same schedules. Oocytes *in vitro* matured by 24h (5,96ρmol BCC; 4,82 ρmol CON), 16h (4.67ρmol BCC; 3,67 ρmol CON) and for 8 hours (4.24ρmol BCC); they present concentration of intracellular GSH significantly higher to the immature oocytes (2.08 ρmol) without thiols addition up to 8h of IVM (3.56ρmol 8HCON). These results demonstrate that the dynamics of the concentrations of GSH was similar with (BCC) or without (CON) thiols addition, except to the 8h of MIV. It remains to know the supplement with thiols in the medium of IVM has impact in the results of *in vitro* development of embryos.

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IN VITRO PRODUCTION OF BUFFALO EMBRYOS – PRELIMINARY RESULTS

Ferraz, M.L.[1]; Watanabe, Y.F.[1,2]; Joaquim, D.C.[1]; Watanabe, M.R.[1]; Accorsi, M.F.[1]; Meirelles, F.V.[2]; Sá Filho, M.F.[3]; Baruselli, P.S.[3]

[1]Vitrogen® Pesquisa e Desenvolvimento em Biotecnologia da Reprodução, Cravinhos–SP, Brasil. [2]Depto. de Ciências Básicas da FZEA-USP, Pirassununga–SP, Brasil. [3]Depto. de Reprodução Animal da FMVZ-USP, São Paulo–SP, Brasil. marcio@vitrogen.com.br

Buffalo breeding in Brazil has gained considerable proportion due to the double productive aptitude (beef and milk), becoming an efficient alternative as a source of animal protein. Superovulation (SOV) for embryo transfer (ET), on the other hand, presents low results in these animals, resulting in a small number of embryos recovered per female. Thus, the association of follicular aspiration and *in vitro* fertilization (OPU-IVF) are an alternative tool to increase embryo production in this species. The aim of this work was to standardize and make usable the embryo production in buffaloes by OPU-IVF and compare the efficiency in this process when using two different reproducers males. Oocytes were obtained by ultrasound guided follicular aspiration using an Aloka SSD 500 transvaginal transducer (Aloka 9111-5, Japan) and selected by their morphology. *In vitro* maturation (IVM) was made in TCM199 + FSH/LH/estradiol + 10% FCS at 38.8°C and 5% CO_2 in air for 24h. Oocytes were then placed in IVF medium (TALP + BSA, PHE and 10μg/ml heparin) with $1x10^6$ motile cells/ml prepared by Percoll gradient (45 and 90%). After 20 to 22 hours the zygotes were cultured in droplets of CR2 medium + 10% FCS in co-culture with granulosa cells up to day 07. Fourteen buffaloes were aspirated, from which 131 viable oocytes (9.35 ± 3.55 oocytes/donor) were recovered and from these a pool of oocytes was made and split between two bulls (A and B), which resulted in a total of 24 embryos (1.7 embryos/female; 18.6% of embryo rate). From these, 18 were from bull A and 6 embryos were from bull B (27.3% and 9.5% of embryo rate, respectively), value significantly superior to Qui-square test ($p<0.0032$). Were evaluated also the eclosion rate of embryos in day 08, when 23 of 24 embryos (96%) were found on this stage. Such results demonstrate the viability of buffalo embryo production *in vitro* and the difference in the results between two bulls used. However, it is necessary to increase the studies with improvements in IVM/IVF/IVC processes to be overcome in order to these techniques to be used.

EVALUATION OF MULTIPLEX-PCR SYSTEM USING INTERNAL CONTROL OF REACTION TO SEX IDENTIFICATION OF *IN VITRO* PRODUCED BOVINE EMBRYOS

Rufino, F.A.[1]; Wosiacki, S.R.[1]; Féres, F.C.[2]; Lisboa, L.A.[2]; Seneda, M.M.[2]; Alfieri, A.A.[1]

[1] Department of Preventive Veterinary Medicine; Laboratory of Animal Virology; [2] Department of Veterinary Clinics; Laboratory of Animal Reproduction - UEL, Londrina-PR, Brazil.
mseneda@uel.br

The protocols carried out commercially for sex determination of embryos involve the use of Y-specific sequence in co-amplification with bovine autosomic sequence. The aim of this work was the development of multiplex-PCR system with internal control of reaction and to evaluate the efficiency in the sex identification of *in vitro* produced bovine embryos. Hole embryos in eclodid blastocyst stages (n=29) were individually transferred to polypropylene tubes with sterile ultra pure water (MiliQ®). For the extraction of embryos DNA they were incubated with proteinase K (Invitrogen™ Life Technologies, USA) for 30min/65ºC and, afterwards, for 10min/95ºC. The multiplex-PCR was carried out using 5µL of extracted DNA and the PCR-mix was constituted by 1x PCR buffer 10x (200 mM tris Hcl, pH 8.4; 500 mM KCl); 2.5 mM of MgCl ; 0,1 mM of dNTP; 2.5 units of Platinum Taq DNA polimerase and sterile ultra pure water (MilliQ®). The reaction was performed with 0,8 pmol of Y-specific primers that amplified a product of 300 base pairs (pb). For the internal control of reaction was used specific primers (0.1 pmol) designed to amplify a 626 pb amplicon from NDR5 gene of mitochondrial DNA of *Bos taurus* and *Bos indicus*. The multiplex-PCR was carried out under the following time and temperature conditions: 5min/95ºC followed by 20 cycles of 1min/95ºC, 90seg/63ºC (annealing of Y-specific primers), 1min/72ºC and 20 cycles of 1min/95ºC, 90seg/63ºC, 30seg/55ºC (annealing of mitochondrial DNA primers) and 1min/72ºC and a final extension cycle of 7min/72ºC. From 29 embryos submitted to multiplex-PCR, 28 embryos (96,55%) had the sex identified. The presence of 2 bands (300 e 626pb) found in 20 embryos (71,42%) identified the male. The presence of only mitochondrial sequence in 8 embryos (28,57%) characterized the female embryos. The use of concomitant mitochondrial DNA amplification secured that no false-female results occurred. In only one sample (3,44%) a lack of DNA amplification suggested a failure in the technique. The amount of primers of mitochondrial DNA was reduced in comparison with Y-specific primers due to very high quantity of mitochondrial DNA contained in the embryos. The reaction was optimized with 40 cycles. The first 20 cycles consisted in the amplification of only Y-specific DNA and, afterwards, it was carried out the amplification of Y-specific and mitochondrial DNA simultaneously. The use of bovine mitochondrial specific primers as internal control of the reaction was of extreme importance considering that the results are more reliable when such procedure was adopted, excluding the possibility of wrong results as false female or absence of amplified product.

OPTIMIZATION OF THE MULTIPLEX-PCR FOR SEX IDENTIFICATION IN IN VITRO PRODUCED BOVINE EMBRYOS

Rufino, F.A.[1]; Wosiacki, S.R.[1]; Lisboa, L.A.[2]; Seneda, M.M.[2]; Alfieri, A.A.[1]

[1] Department of Preventive Veterinary Medicine; Laboratory of Animal Virology; [2] Department of Veterinary Clinics; Laboratory of Animal Reproduction - UEL, Londrina-PR, Brazil.
mseneda@uel.br

Deviation has been observed in the ratio of male: female sex of in vitro produced embryos with the percentage of male embryos superior to the theoretical 50% for each sex. The lack of recipients in sufficient number and quality, added to the inadequacy of freezing in vitro produced bovine embryos enhances this disproportion. This fact opens perspectives to the installation of an effective system to determine the sex of these in vitro embryos. The aim of this work was the optimization of multiplex-PCR protocol for sex identification of in vitro produced embryos. Sixty-three embryos in blastocyst and eclodid blastocyst stages were individually transferred to polypropylene tubes containing 10 µL of sterile ultra pure water (MilliQ®). In each tube, 0.8 µL of proteinase K (Invitrogen ™ Life Technologies, USA) was added and incubated for 30 minutes at 65°C and afterwards, for 10 minutes at 95°C for embryonary cell lysis. The multiplex–PCR was performed with 5 µL DNA extracted and sterile ultra pure water (MilliQ®) and 45 µL of PCR – mix constituted by 1x PCR buffer 10x (200 mM tris Hcl, pH 8,4; 500 mM KCl) (Invitrogen TM Life Technologies, USA); 2.5 mM of MgCl2; 0.1 mM of dNTP; 2.5 units of Platinum Taq DNA polimerase; 0.8 pmol especific oligonucleotides primers designed to amplify 300 pb amplicon from bovine Y chromosome and 0.1 pmol of internal control primers designed to amplify 626 pb amplicon from bovine mitochondrial DNA and sterile ultra pure water (MilliQ®) for final volume of 50 µL. The reaction was performed with the following time and temperature conditions: 5 min/95°C followed by 20 cycles of 1min/95°C, 90 sec/60°C (annealing of Y–specific primers), 1 min/ 72ºC and 20 subsequent cycles with 1 min/95ºC, 90 sec/63°C, 30 sec/55°C (annealing of autosomic primers) and 1 min/72°C completing 40 total cycles and a final extension of 7 min/72°C. The technique efficiency was 90.47% (57/63). The presence of two bands, 626 pb and 300 pb amplicon, shown in 38 embryos (60.31%) determined a male embryo. Nineteen embryos (30.15%) were considered female in which only the autosomic sequence was observed (626 pb amplicon). The results suggested a successful multiplex–PCR practical protocol for sex identification of in vitro produced embryos.

CUMULUS-OOCYTE INTERACTION IN PROGRAMMED CELL DEATH IN BOVINE

Emanuelli, I.P.[1]; Carambula, S.F.[1]; Ripamonte, P.[1]; Mesquita, L.G.[1]; Biase, F.[1]; Perna Junior, F.[1]; Leal, C.L.V.[1]; Santos, W.K.F.[1]; Merighe, G.K.F.[1]; Meirelles, F.V.[1]

[1]Departamento de Ciências Básicas – Laboratório de Morfofisiologia Molecular e Desenvolvimento, FZEA/USP, Pirassununga-SP, 13635-970 – Brazil. isabele@cesumar.br

It is known that the *in vitro* embryo production is influenced by several factors, among them, the morphological quality of cumulus-oocyte complexes (COCs). Considering the heterogeneity of COCs recovered from abattoir derived ovaries (1), the interaction between oocytes and cumulus cells (CC) (2) and the observation of programmed cell death in CC (3), the present work aimed to study the cumulus-oocyte interaction on the process of programmed cell death in bovine COCs of different morphological classes. The *COCs were obtained from* bovine ovaries and classified according to the morphology of their cumulus cell layers, as follows: class A, compact and with many layers; class B, compact with few layers; class C, expanded (all classes with homogeneous ooplasm). Before *in vitro* maturation (IVM), *DNA fragmentation in cumulus cells (CC) was evaluated.* CC (0h and 24h after IVM) *were analyzed for BCL-2* and *BAX* transcripts and proteins. *The results showed that the majority of class A immature oocytes harbor minimal or inexistent DNA fragmantation, contrasting with the other classes of oocytes. I*mmature class B and C COCs, have the most frequent *DNA fragmentation (A:* 0.0%; B: 5.98%; C: 7.78%; $p<0.001$). *After IVM, an increase in CC DNA fragmentation was observed in B and C COCs, mainly in B group (A: 4.06%; B: 39.45%; C: 13.44%; $p<0.001$). BCL-2* protein in CC had the same expression level in all COCs groups. However, the BCL-2/BAX proteins ratio was higher in A and C groups. COC-B had the highest *BAX* expression and lower ratio. *These data demonstrate that that the lower the BCL2/BAX protein ratio is the greater the DNA fragmentation in CC, although this relation does not occur with transcripts.*

KINETIC COMPETENCE OF COCS DURING IN VITRO CULTURE TO THE BLASTOCYST STAGE

Emanuelli, I.P.[1]; Carambula, S.F.[1]; Ripamonte, P.[1]; Mesquita, L.G.[1]; Perna Junior, F.[1]; Cortezzi, S.S.[1]; De Bem, T.H.C.[1]; Merighe, G.K.F.[1]; Leal, C.L.V.[1]; Meirelles, F.V.[1]

[1] Departamento de Ciências Básicas, Universidade de São Paulo, Pirassununga, São Paulo, Brazil, 13635-900. isabele@cesumar.br

About 40% of fertilized bovine oocytes do not complete development during the preimplantation period. It is known that the in vitro embryo production system is influenced by several factors, among them, the morphological quality of cumulus-oocyte complexes (COCs). These factors could lead to alterations in oocyte maturation and resulting in variable developmental rate. Thus, the aim of this study was to evaluate the effects of morphological classes of bovine COCs on oocyte nuclear maturation and embryonic development. The cumulus oocyte-complexes *COCs (n=650) were collected from slaughtered cows'* ovaries and classified according to the morphology of their cumulus cell layers, as follows: class A, compact and with many layers; class B, compact with few layers; class C, expanded (all classes with homogeneous ooplasm). The COCs were matured *in vitro* in bicarbonate-buffered TCM-199 supplemented with FCS. Following maturation, the oocytes were parthenogenetically activated (5μM ionomycin + 2mM 6-DMAP) *and* cultured in SOF medium at 38°C in 5% CO_2 and 5% O_2. T*he hatching rate and quality of parthenogenetic embryos at the 9th day post-activation were also* evaluated. The group COC-A reached the fourth cell cycle faster in comparison with groups COC-B and COC-C, moreover, oocytes derived from COCs of A quality showed greater ability *to reach 8-cell stage* (COC-A - 41.7%; COC-B – 27.5% e COC-C – 33.3%; $p<0.05$). COC-A selection for *in vitro* embryo production was positive for cleavage rate, as well as in the blastocyst and hatching rates at D9. Lower rates ($p>0.05$) for cleavage and blastocyst production were obtained in groups B and C compared with group A. *However, when 8-cell/blastocyst rates were evaluated, n*o difference was observed *between groups* (A– 41.1%; B– 30.5%; C– 32.6%; $p>0.05$). *The morphological type of COCs* also did not change *blastocyst quality. These data demonstrate that the morphological type of COCs was not related with blastocyst quality, but affected the proportion of embryos capable of overcoming developmental block and reaching the blastocyst stage.*

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COMMERCIAL *IN VITRO* PRODUCTION OF OVINE EMBRYOS

Watanabe, M.R.[1]; Joaquim, D.C.[1]; Meirelles, F.D.P.[2]; Meirelles, F.V.[2]; Watanabe, Y.F.[1,2]

[1]Vitrogen® Pesquisa e Desenvolvimento em Biotecnologia da Reprodução, Cravinhos–SP, Brasil.;
[2]Depto de Ciências Básicas da FZEA-USP, Pirassununga-SP, Brasil.
michele@vitrogen.com.br

In the last few years, Brazilian ovine breeding has presented a great interest in developing new reproductive techniques aiming the fast spread of animals of high genetic potential. Since the birth of several lambs 15 years ago, advances have been observed in the different steps of embryo *in vitro* production, from oocyte maturation to embryo culture conditions. Although IVF results in sheep in Brazil are still preliminary, it is important to dominate these techniques of embryo *in vitro* production, especially regarding oocyte recovery and sperm capacitation. The aim of this work was to assess the variation in embryo production when four different rams were used in commercial settings. Oocytes were obtained by laparoscopic follicular aspiration (LOPU), selected according to their morphology and later *in vitro* matured in TCM199 plus hormones (FSH/LH/estradiol) and 10% FCS 24 h at 38.7°C and 5 % CO_2 in air. Motile sperm cells were obtained by centrifugation in different Percoll gradients (45 and 90%) and fertilized in TALP medium with 10μg heparin/ml and at a concentration of $1X10^6$ sperm cells/ml. After 24h incubation, zygotes were transferred to *in vitro* culture medium CR2 + 10% FCS. After 7 days, embryos were evaluated and classified according to their developmental stage for later transfer to synchronized recipients. Data were analyzed by ANOVA and compared by TUKEY-KRAMER HSD test. Three rams were assessed mated with 11 donors in 13 LOPU sessions, where 107 viable oocytes were obtained, 32 embryos (30%), from which 7 were transferred resulting in 1 pregancy at 30 days (14%). Another 15 embryos were transferred and are still waiting for pregnancy diagnosis. According to the results, a significant variation ($P<0.05$) among rams was observed regarding blastocyst development rates (R1: 15.9±8.0%, R2: 57.5±10.3% and R3: 25.1 ± 8.0%). Ram 1 (R1) mated with 5 donors obtained a variation in embryo production of 10 to 22%, R2 with 2 donors showed 53 and 67% embryos and R3 with 4 donors had 0 to 64% embryo production. It is concluded that there is an individual variation among rams regarding embryo production (morulae and blastocysts). However, it is important to test the effect of the matings on embryo development.

The present work had the collaboration of the Genoma Animal and Biomax teams in oocyte recovery by LOPU.

EFFECT OF HEPARIN IN BOVINE IN VITRO CULTURE ON DEVELOPMENTAL RATE AND SEX RATIO

Pegoraro, L.M.C.[1]; Rheingantz, M.G.T.[2]; Bredbacka, P.[3]; Coscioni, A.[4]; Rambo, G.[5]; Abrahão, M.[6]; Meirelles, M.[6]; Saalfeld, M.H.[7]; Agnonni, L.[1]

[1-]Embrapa Clima Temperado Br 392 km 78 Pelotas 96001-970 RS, Brasil; [2-]Inst. Biologia, UFPel; [3-]Finnenzymes, Finlândia; [4-]PPGZ-FAEM UFPel; [5-]Bolsista IC UFPel; [6-]Estagiários Laboratório de Reprodução Animal Embrapa Clima Temperado [7-]Emater-RS. ligia@cpact.embrapa.br

The sex ratio deviation of bovine in vitro produced embryos is described in several studies. Many factors can be involved in this process including components of the culture media. The objective of the experiment was to test the effect of heparin in different concentrations (0, 5, 10 and 20 µg/mL) in the culture medium on developmental rates and sex ratio.. Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory in physiologic solution supplemented with antibiotics at the temperature of 25°C. Cumulus-oocyte complexes were maturated in TCM 199 medium with pyruvate, FSH/LH and penicillin and streptomycin during 24 h in 5% CO_2 at 38.5°C. After maturation, the oocytes were transferred to fertilization medium (IVF-TALP) in which they were inseminated with spermatozoa prepared by the swim-up method. After 18 h, the oocytes were vortexed for 2 minutes to separate the cumulus cells. The presumable zygotes were cultured in SOFaa medium supplemented with 0.5% FBS, pyruvate, gentamicin and different heparin concentrations in 5% CO_2 at 38.5°C for 7 to 8 days. Four culture groups were used: G 1 (n = 178, control, without heparin), G 2 (n=189, 5 µg/mL heparin), G 3 (n=182, 10 µg/mL heparin) and G 4 (n=193, 20 µg/mL heparin). The embryonic development in the different groups, measured by viable blastocysts proportion, was evaluated on Day 7 of culture. The results of the experiment were analyzed by the Chi-square test. The developmental rates (% blastocysts/cleaved) in D-7 were higher in control group then other treatments (51.2 versus; 29.7; 25.6; 23.3; $P<0.001$). After the PCR sexing of the embryos, it was observed that the percentage of males in the control group differed from the expected 50% (66.15%, $P<0.05$). A tendency ($P <0,1$) was observed for smaller deviation in sex ratio when embryos were cultured in the presence of heparin at the concentration of 20 µg/mL (48,72% males). These results demonstrate the toxicity of heparin under the chosen culture conditions. The shift of the sex ratio suggests that female embryos are more tolerant to the harmful effects of heparin.

INFLUENCE OF SUPERESTIMULATION AND HORMONAL DEPRIVATION PROTOCOLS ON *IN VITRO* PRODUCTION OF NELORE EMBRYOS (*Bos taurus indicus*)

Satrapa, R.A.[1]; Ferreira, M.M.G.[2]; Ederhardt, B.G.[2]; Monteiro, F.M.[2]; Melo, D.S.[2]; Potiens, J.R.[3]; Barros, C.M.[2]

[1]Departamento de Reprodução Animal e Radiologia Veterinária – FMVZ/UNESP. [2]Depto. de Farmacologia, IB-UNESP, Botucatu. [3]Central Bela Vista, Pardinho- SP. rsatrapa@yahoo.com.br

There are indications in the literature that delaying the period between ovarian superestimulation and ovum pick up (OPU) would induce follicles to a condition of initial atresia, which could be beneficial to oocyte development. Blondin et al. (*Biol Reprod* 66: 38-43, 2002) superstimulated (FSH) Holstein heifers and delayed OPU for 48 h, i.e., they induced "initial atresia" in these follicles deprived of FSH (starvation) for 48h. Additionally, 6h before OPU, LH was administered to accelerate follicular maturation. This protocol yielded a surprisingly high blastocyst rate (80.4±9.4%). In the present work Blondin's protocol was simultaneously compared to other protocols used for OPU and *in vitro* production of embryos (IVP), in Nelore cattle. Nelore cows (n=18) were randomly allocated in 3 groups: Group 1 (just OPU), Group 2 (superestimulation and OPU), and Group 3 (superestimulation associated with FSH deprivation and OPU). Three OPU were performed, and the animals were switched to a different group each time, in such a way that at the end of the experiment all cows received the 3 protocols. At random stage of the estrous cycle (D-2), follicles =6 mm were aspirated to induce a new follicular wave 2 days afterwards (D0). In Group 1, OPU was performed on D2 and oocytes were processed to IVP. In Group 2, starting on D0, cows were superestimulated (pFSH, Folltropin®, 30 mg administered daily, i.m., during 3 consecutive days), and 6 h after the last FSH dose they received exogenous LH (12.5 mg, i.m., Lutropin®, D3). OPU was performed 6 h after LH administration, i.e., 12 h after the last dose of FSH. Animals in Group 3 received the same treatment as in Group 2, except that LH was administered 42 h after the last dose of FSH, and OPU occurred 6 h later. Therefore, in this Group, follicles were deprived of FSH during 48 h. Both clivage and blastocyst rates were similar ($p>0.05$, Logistic Regression) among oocytes from Groups 1, 2 and 3, respectively: 77.4% (144/185) and 42.70% (79/185); 75.54% (105/139) and 31.65% (44/139); 63.52% (101/159) and 33.33% (53/159). However, hatched blastocyst rate was higher ($p<0.01$) in Group 1 (30.27%, 56/185) when compared to Grupo 2 (11.51%, 16/139) or 3 (15.72%, 25/159). It is concluded that, contrary to previous work on European breeds (taurus), ovarian superestimulation associated with deprivation of FSH and OPU (Group 3) do not increase IVP of Nelore embryos (indicus). Additionally, the highest hatched blastocyst rates were observed in oocytes from non superstimulated cows (Group 1).

PROGESTERONE AND OR ESTRADIOL INCREASES THE NUMBER OF CELLS IN BOVINE *IN VITRO* PRODUCED BLASTOCYSTS

Reis, A.N.[1]; Miranda, M.S.[2]; Silva, A.O.A.[1]; Sousa, A.J.O.[1]; Silva, L.K.X.[1]; Sousa, J.S.[1]; Ohashi, O.M.[2]

[1]Central de Biotecnologia de Reprodução Animal – UFPa, Castanhal-Pa, [2]Laboratório Fertilização *in vitro* – UFPa, Belém-Pa. areis@ufpa.br

The objective of this work was to evaluate the number of cells in the bovine blastocysts, co-cultured with bovine oviductal epithelial cells. BOECs were exposed or not to estradiol and progesterone (P_4) during in vitro culture. The ovaries were collected at a slaughterhouse and selected COCs were allocated in IVM medium (TCM-199 with Bicarbonate supplemented with hormones, serum and antibiotics), for 24 h at 38,5°C in 5% CO_2 atmosphere, supplement or not with estradiol (1 µg/ml). Frozen-thawed semen was centrifuged in a Percoll gradient (45 and 90%) during 30 min at 200 x *g*. The final concentration was of 10^6 spermatozoa/ml in IVF medium (TALP supplemented with PHE, Heparine and BSA). After fertilization, The BOECs were washed and added to different groups in CR_2 medium, supplemented with or without P_4. Group 1 (CR_2 + P_4-100µg/ml and BOECs + Estradiol); Group 2 (similar to the G1, without P_4); Group 3 (CR_2 + P_4-100 µg/ml, and BOECs without exposition to Estradiol) and Group 4 (CR_2 + BOECs). Data were submitted to ANOVA ($P<0,05$) and Tukey test. The embryonic quality was determined by the total number of cells of the blastocysts, being observed 10 blastocistos of each group, through to the HOECHST 33342. It was observed that when the BOECs was associates to estradiol and/or progesterone (G1, G2 and G3) the number of cells of the blastocysts increased significantly ($P<0.05$) (125.9; 128.4 and 123.4 cells, respectively) when compared with the G4 group that only contained the BOECs (112.5 cells) without hormonal stimulation. We conclude that the addition of estradiol and progesterone, separately or in combination, in co-culture of CEOB demonstrated significantly to increase the number of cells of the blastocysts when compared with the group that only contained cells of oviduct.

BACTERIAL CONTAMINATION IN LIQUID NITROGEN CONTAINERS

Rubin, M.I.B.[1]; Silva, C.A.M.[1]; Oliveira, R.A.[1]; Pessoa, G.A.[1a]; Navarro, R.B.[1a]; Pretto, A.N.[2b]; Vargas, A.C.[2]

[1]Embryolab – Laboratory of Animal Embryology – Department of Large Animal Clinics, Building 97 B.4 South Area – Veterinary Teaching Hospital; [2] Microbiology Laboratory – Department of Preventative Medicine Building 44 North Area/Agricultural Center
[a ,b] Graduate Students in Veterinary Medicine. Federal University of Santa Maria 97.105-900 Santa Maria/RS, mararubin@smail.ufsm.br

The storage of semen and embryos in liquid nitrogen (N_2) is a very common form of gene preservation and genetic improvement of the herd. Handling the N2 containers must be adequate. Inappropriate care of the containers using poor hygiene habits or inadequate manipulation of the container techniques may result in contamination that could negatively affect the artificial insemination or embryo transfer process. There are not many publications in the literature about what kind of pathogens live inside the N_2 containers, especially those coming from distant farms when the AI or TE season is over. In this study swabs were collected from two canes still under the liquid N_2 and from the bottom of the container (n=8), used for storage of semen and embryos from different farms located at the central region of the state of Rio Grande do Sul. The samples were identified and sent immersed in a modified Stuart's transport medium to the Microbiology Laboratory of the Federal University of Santa Maria (LABAC) to be cultured for aerobic bacteria. Afterwards the canisters and containers were washed with extran soap (Merck) and disinfected with 2% glutaraldehyde solution (Glutaron II® - Rioquímica) and 70% ethanol. They were left to dry at room temperature and a second sample was taken from each container and canisters. The *Bacillus* cereus was isolated from eight containers as well as from corresponding canisters. In two containers a Gram-negative was isolated. After the disinfections was performed none of the containers showed evidence of bacterial contamination. This indicates the importance of regular maintenance and disinfection procedure should be taken in account to ensure the correct preservation of the genetic material used in artificial insemination and embryo transfer programs.

ANALYSIS OF TRANSCRIPTS ASSOCIATED TO *DE NOVO* DNA METHYLATION AND CHROMATIN STRUCTURE IN BOVINE BLASTOCYSTS

Camargo, L.S.A.[1]; Powell, A.M.[2]; Vale Filho, V.R.[3]; Wall, R.J.[2]

[1]Embrapa Dairy Cattle, Juiz de Fora, MG, Brazil. 36038-330. [2]Biotechnology and Germplasm Laboratory, BARC, USDA, Maryland, EUA. 20705. [3]Veterinary School, UFMG, Belo Horizonte, MG, Brazil. 30123-970. camargo@cnpgl.embrapa.br

DNA methylation and chromatin structure play an essential role on regulation of gene expression, and are important for imprinting maintenance. During early embryo development, DNA undergoes an extensive demethylation, followed by *de novo* methylation following the morula stage of development. Modifications in DNA methylation pattern and/or on histone acetylation/deacetylation in *in vitro* cultured or somatic cell nuclear transfer embryos may cause epigenetic alterations that contribute to the low efficiency of *in vitro* fertilization and nuclear transfer. The aim of this study was to evaluate the expression of genes that encode proteins associated with *de novo* DNA methylation (DNA methyltransferase-3A and -3B) and histone acetylation (histone acetyltransferase 1 and GCN5) and deacetylation (histone deacetyltransferase 1) in bovine blastocysts produced *in vivo*, *in vitro* (IVF) and by somatic cell nuclear transfer (SCNT). *In vivo*-produced blastocysts were collected from superovulated cows, seven days after artificial insemination. SCNT embryos were produced with serum-starved fibroblast cell obtained from 3 months-old fetuses muscle. IVF zygotes and SCNT embryos were cultured for 3 days in G1.1 medium, followed by 4 days in G2.2 medium, both without serum and under 5% CO_2, 5% O_2 and 90% N_2 at 38.5ºC. IVF and SCNT blastocysts were recovered at 180-186h after *in vitro* fertilization or activation, respectively. Embryos were frozen and stored in liquid N_2 until RNA extraction. Extracted RNA was amplified and all genes were analyzed in individual embryos. Ten *in vivo*-produced, 14 IVF and 14 SCNT blastocysts were evaluated. Relative quantification was performed using Real Time PCR, in duplicate, and the results were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, used as internal reference gene. Statistical analysis was performed by ANOVA. All genes analyzed were more highly expressed ($P<0.01$) in *in vivo*-produced blastocysts than for IVF and SCNT blastocysts. No differences ($P>0.05$) in expression were detected between IVF and SCNT blastocysts. These results suggest that the components required for chromatin remodeling are reduced in embryos produced in vitro (IVF and SCNT blastocysts). Thus, it would appear that the environment in which the embryo develops has a more profound influence on gene expression than the method by which the embryo was produced.

IN VITRO PRODUCED BOVINE EMBRYOS CULTURED IN CR2 MEDIUM SUPPLEMENTED WITH KNOCKOUT™ SR

Serapião, R.V.[1,4]; Ramos, A.A.[2]; Polisseni, J.[1]; Folhadella, I.M.[1,3]; Jasmin.[1]; Camargo, L.S.A[1]; Viana, J.H.M.[1]; Ferreira, A.M.[1]; Sá, W.F.[1]; Fonseca, F.A.[4]

[1]Embrapa Gado de Leite - Juiz de Fora - MG; [2]Empresa de Pesquisa Agropecuária de Minas Gerais - Epamig – Belo Horizonte - MG; [3]Escola de Veterinária – UFMG – Belo Horizonte – MG; [4]Universidade Estadual do Norte Fluminense Darcy Ribeiro – UENF – Campos dos Goytacazes – RJ
rserapis@yahoo.com.br

Knockout™ SR (KSR; Gibco Labs., Grand Island, NY) is a serum replacer for culture media, optimized to support embryonic stem cells in culture. The aim of this study was to evaluate the effect of KSR on development of *in vitro* fertilized bovine embryos, since serum has been associated to many alterations in *in vitro*-generated bovine embryos. Oocytes obtained from slaughterhouse ovaries were *in vitro* matured in TCM 199 medium (Gibco Labs, Grand Island, NY), supplemented with 10% Estrus Calf Serum (ECS) and 20μg/ml FSH, for 24 hours. After maturation, oocytes were *in vitro* fertilized with frozen-thawed semen from a bull previously selected. Viable spermatozoa were obtained by swin up method. Fertilization was performed with 2,0 x 10^6 sperm/ml in Fert TALP medium supplemented with heparin, for 22 hours. Presumptive zygotes were randomly divided in three treatments, T1 (n=389): CR2aa supplemented with 10% fetal calf serum (FCS); T2 (n=426): CR2aa supplemented with 10% KSR, and T3 (n=424): CR2aa supplemented with 3mg/ml PVA. Embryos were co-cultured with their own *cumulus* cells in 50μl drops, covered with mineral oil. All steps were performed at 38,5 °C, under 5% CO_2 in air and 95% humidity. Cleavage rate was evaluated at 72 hours post-fertilization; blastocyst rate at day seven, and total cell number at day eight post-fertilization. Cleavage and blastocyst rates were analyzed by chi-square and total cell number by analysis of variance, with embryo development stage as covariant. No difference was observed among T1, T2 and T3 ($P>0.05$) on cleavage rate (68.9%, 71.4% and 67.2% respectively). However, blastocyst rate in T2 (12.4%) was lower than T1 (19.2%) and higher than T3 (5.2%). Blastocysts in T1 and T2 presented similar ($P>0.05$) total cell number (109.4±6.1 and 105.9±5.9, respectively), but greater ($P<0.01$) than blastocysts in T3 (79.6±8.4). KSR provided lower blastocyst rate when compared to FCS, however the total cell number was not affected. KSR supported greater blastocyst development rate than PVA. In conclusion, KSR is able to support development of *in vitro* fertilized bovine embryos and it can be an alternative when serum-free culture medium is recommended.

CULTURE OF *IN VITRO* PRODUCED BOVINE EMBRYOS IN POLIVINYL ALCOHOL (PVA)

Silva, D.S.[1]; Pozzobon, S.E.[1]; Navarro, R.B.[1]; Melo, L.C.[1]; Silva, C.A.M.[1]; Rubin, M.I.B.[1]

[1]Embryolab – Laboratory of Animal Embryology – Departament of Large Animal Clinics Building 97 B.4 South Área – Veterinary Hospital – Agricultural Center; Federal University of Santa Maria 97.105-900 Santa Maria/RS; sepozzobon@yahoo.com.br

Reliable protocols for *in vitro* production (IVP) of bovine embryos are necessary, besides maintaining the health status of the herds, to yield satisfactory results, constant embryo production rates and healthy newborn calves. In order to evaluate the effect of 0.1% polyvinyl alcohol (PVA) in the *in vitro* culture medium, *Cumulus* oocyte complexes (COC) were matured for 24h in 4-well Nunc dishes with TCM-199 supplemented with 10% EMS (estrus mare serum; Control group), 0,025mg/mL of sodium pyruvate, 2.2mg/mL $NaHCO_3$, 0.01UI rFSH-h/mL, 0.5µg/mL LHb in an atmosphere of 5% CO_2 in air at 39ºC. Semen for fertilization (D0) was selected by Percoll density gradients, capacitate with heparin and hiperactivated with PHE, and concentration adjusted to $1x10^6$ spermatozoa/mL. COC were placed in 4-well Nunc dishes with 400µL Fert-Talp medium for 18-20h and incubated with spermatozoa at 5%CO_2 in air. Zygotes were cultured in 4-weel Nunc dishes in SOFaaci with 0.1% PVA or 5% EMS under mineral oil. The plates with oocytes were placed in a plastic bag where a gaseous mixture was injected (5% CO_2 5% O_2 and 90% de N_2). Cleavage rates (D2) evaluated 48h after fertilization did not differ and reached 89.1% (193/217) with PVA and 91.6% (196/214) with EMS. Blastocyst production in D7 and hatching rates in D9 were similar, respectively, with PVA (28.5% and 22.1%) and EMS (29.9% and 24.2%). It was concluded that replacement of EMS by PVA in the culture medium results in similar rates of embryo production.

Key words: embryo, PIV, medium defined, PVA.

EVALUATION OF DNA FRAGMENTATION AFTER FERTILIZATION IN BOVINE EMBRYOS MATURED *IN VITRO* IN MEDIA SUPPLEMENTED WITH DIFFERENT SOURCES OF MACROMOLECULES

Lo Turco, E.G.[1]; Barretto, L.S.S.[1]; Perecin, F.[1]; Soria, G.[1]; Bertolla, R.P.[2]; Mingoti, G.Z.[3]

[1]Departamento de Reprodução Animal, FCAV – UNESP, Jaboticabal; [2]Setor de Reprodução Humana Hospital São Paulo, UNIFESP, São Paulo; [3]Departamento de Produção e Saúde Animal, FMVA – UNESP, Araçatuba

Evaluation of double-strand DNA fragmentation has been shown to be a satisfactory means of evaluating bovine embryo quality in various stages of development. This study was set out to analyze double-strand DNA fragmentation in bovine embryos produced after fertilization of *in vitro* matured oocytes in media supplemented with three different types of macromolecules: fetal bovine serum (FBS), bovine serum albumin (BSA), these two chemically undefined, or polyvinyl alcohol (PVA), a chemically defined macromolecule. Ovaries were collected at a slaughterhouse, and oocytes were aspirated and matured for 24 hours in maturation culture media (TCM-199, 0.2mM sodium piruvate, 25mM sodium carbonate, 75µg/mL Kanamycin, 1µg 17ß-Estradiol/mL, 0.5µg FSH/mL, 100 IU hCG/mL), supplemented with either 1% PVA, 1% BSA, or 1% FBS, according to the experimental group, and incubated at 37°C in a 5%CO_2 in air atmosphere. Following fertilization, all the zygotes were cultured in a same media until they reached the blastocyst stage (144 hours after fertilization), and were subsequently submitted to the TUNEL (terminal deoxynucleotidyl transferase mediated UDTP nick-end labeling). DNA fragmentation rates were 2.15%±0.468 for the FBS group, 1.75%±0.199 for the BSA group, and 1.80±0.470 for the PVA group. No significant differences were found ($P>0.05$). The type of macromolecule added to the maturation culture media does not influence double-strand DNA integrity in bovine embryos cultured to the blastocyst stage, but many other mechanisms regarding the impact of these macromolecules on oocytes during *in vitro* maturation remain to be studied.

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APOPTOSIS EVALUATION OF *IN VITRO* PRODUCED PIG EMBRYOS (PARTIAL RESULT)

Coutinho, A.R.S.; Simões, R.; Marques, M.G.; Nascimento, A.B.; Oliveira, V.P.; Tavares, M.N.; Visintin, J.A.; Assumpção, M.E.O.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, nanicout@usp.br

Apoptosis is a physiological event involved with death and tissues replication, demonstrating an important function on tissues organization during embryogenesis. This mechanism occurs in vivo as well as in vitro in pre-implantation embryos, but most frequent in the latter. The transcriptional activation in pig embryos occurs in four-cell cycle, which is the longer cell cycle stage during the pre-implantation period, characterized by the embryonic developmental blockage that increases embryos loss. The aim of this study was to evaluate a correlation between the apoptosis mechanism and developmental blockage of *in vitro* produced pig embryos. These were produced from immature oocytes after IVM/ IVF and IVC in PZM-3 medium, containing 3mgBSA/ml at 38,5°C, 5%CO_2 in air and humidified atmosphere at 95%. The embryos development have been analyzed 48hs after the culture beginning in order to verify cleavage, blockage, non-blockage and fragmented embryos rates. Out of 625 grade I, II, III oocytes to PIV, 0.7±0.05 (430/625) cleaved; 0.27±0.1 (166/625) underwent blockage; 0.43±0.1 (264/625) developed further to blockage and 0.29±0.05 (195/625) showed fragmentation. Blocked and non-blocked embryos were assessed to evaluate apoptosis rate. A qualitativr assay allowed apoptosis estimation by fluorescence of embryos cells. DNA stained by YOPRO-1, permeable at plasmatic membrane in apoptotic cells in the early stage was indicative of apoptosis. The embryos were stained with 1μM YOPRO-1/500 μl PBS and incubated 15 minutes at 38,5°C, 5% CO_2 in air and humidified atmosphere at 95% being immediately observed with confocal microscopy. The blocked embryos showed a positive stain to apoptosis, non-blocked embryos showed no nuclear staining. In conclusion, the developmental blocked embryos suffer apoptosis, although it is important to point that specific molecular apoptosis assays must be performed to confirm this result.

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CHROMOSOMAL ABNORMALITIES AT EARLY STAGES OF DEVELOPMENT IN ZEBU EMBRYOS PRODUCED *IN VITRO**

Pires, R.M.L.[1]; Alvarez, R.H.[1]; Ligori, A.C.[1]; Coelho, L.A.[1,2]; Martinez, A.C.[1]

[1]Centro de P&D Genética e Reprodução Animal, Instituto de Zootecnia, Rua Heitor Penteado, 56, Centro, Nova Odessa, SP, 13460-000; [2]Fac. de Zootecnia e Engenharia de Alimentos, USP, Av. Duque de Caxias Norte, 225, 13635-900, Pirassununga, SP. pires@iz.sp.gov.br

Bovine embryos produced in vitro (IVF) have a high incidence of chromosomal anomalies. However, such occurrence still was not determined in zebu embryos. The aim of the present study was to evaluate the frequency of chromosomal alterations of IVF zebu embryos. It was used IVF embryos of 2, 4, 8 and 16 cells and compacted morulae derived from oocytes collected from ovaries of slaughtered Nelore cows. Morulae recovered from superovulated Nelore cows (IVP) were used as control. Embryonic cells were submitted to conventional procedures (Giemsa staining) and karyotype reading with an optical microscopy. Differences between normal and abnormal karyotype was established using χ^2 test. It was found chromosomal anomalies in 29.9% (32/107) of IVF embryos and 13.0% (3/23) of IVP embryos (P>0.05). Also, incidence of chromosomal anomalies was not influenced by the stage of development of IVF embryos. Mixoploidy and polyploidy were the only chromosomal anomalies present in both, IVF and IVP embryos. Chromosomal anomalies were presented in 40.6% and 59.4% of male and female embryos, respectively (P>0.05). It was concluded that IVF zebu embryos at early stages of development present a high proportion of chromosomal anomalies and their incidence is not related to a specific period of embryonic development.

EFFECT OF CR2aa, KSOMaa and SOFaa IN THE CULTURE OF IN VITRO PRODUCED BOVINE EMBRYOS

Tavares, L.M.T.; Feitosa, W.B.; Mello, M.R.B.; Lima, A.S.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, visintin@usp.br

The objective of this study was to verify the semi-defined chemically media CR2, KSOM and SOF supplemented with amino acids (aa) and 0,3 mg of BSA/ml on the development of in vitro fertilized bovine embryos till blastocyst stage. Oocytes were *in vitro* matured (TCM199 + 10%FCS + HCG + FSH + 17β estradiol) under 5%CO_2 in air and humidified atmosphere at 38.7°C for 24 hours. The semen was thawed, centrifuged in Percoll gradient (45/90%) and diluted at final concentration of $1x10^6$ spermatozoa/ml. For IVF (D0), the gametes were cultivated (sp-TALP + PHE + heparin) under 5%CO_2 in air and humidified atmosphere at 38.7ºC for 18 hours. The presumable zygotes were cultured in 500µl of the semi-defined chemically media CR2aa (n= 307), KSOMaa (n= 279) or SOFaa (n= 241) under 5%CO_2 in air and humidified atmosphere at 38.7ºC, during 10 consecutive days. It was evaluated the rates of cleavage at day 3, morulae at day 7 and blastocyst and hatching blastocyst at day 10. The data were analyzed by Chi-square test (X^2 at 5% significance). The results showed that there were no differences (P>0,05) on cleavage rates among media CR2aa (73.3%), KSOMaa (78.5%) and SOFaa (71.8%). About morulae rates no significant differences were observed among SOFaa and CR2aa or KSOMaa media (2.9% x 5.3%; 7.3%). In relation to early blastocyst (eB) and blastocyst (Bl) rates a significant difference (P<0,05) was observed among SOFaa and CR2aa or KSOMaa media (eB = 0.6% x 3.1%; 3.2% and Bl = 2.9% x 4.6%; 6.2%). Analyzing hatching blastocyst rates, CR2aa (58.7%) showed significant difference when compared to SOFaa (73.7%) medium, but did not differ from KSOMaa (65.2%). In conclusion, CR2aa and KSOMaa media showed better conditions for embryonic development, reaching blastocyst stage.

CULTURE OF IN VITRO PRODUCED EMBRYOS IN SEMI-DEFINED MEDIA OR IN CO-CULTURE OF PRIMARY CELLS

Tavares, L.M.T.; Feitosa, W.B.; Magnusson, V.; Goissis, M.D.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, visintin@usp.br

Pregnancy rates of in vitro derived embryos are not constant. This variation is related to embryo quality that is influenced by different culture systems. The aim of this study was to evaluate the development of *in vitro* produced bovine embryo and cultured in chemically semi-defined media CR2, KSOM and SOF added of aminoacids (aa) and 10%FCS or co-cultured in TCM199 medium with granulosa (GC) and oviductal (OC) cells with 10%FCS. Oocytes derived from slaughthterhouse ovaries were in vitro matured (TCM199 + 10%FCS + HCG + FSH + 17ß estradiol) under 5%CO_2 in air and humidified atmosphere at 38.7(C for 24 hours. For the IVF, semen was thawed, centrifuged in Percoll gradient (45/90%) and diluted at final concentration of $1x10^6$spermatozoa/ml. The oocytes were fertilized (DO) in IVF medium (sp-TALP + PHE + heparin) under 5%CO_2 in air and humidified atmosphere at 38.7ºC for 18 hours. The presumable zygotes were cultured in 500µl of chemically semi-defined media CR2aa (N= 307), KSOMaa (N= 304) or SOFaa (N= 302) or co-cultured in TCM199 with GC (N= 901) or OC (N= 653) at 38.7ºC under 5%CO_2 in air and humidified atmosphere, during 10 consecutive days. Cleavage rate was evaluated at day 3, morulae/blastocyst at day 7 and hatching at day 10. The data were analyzed by ANOVA. No significant differences were observed ($p>0.05$) on cleavage rates among CR2aa (67.1%), KSOMaa (77.6%), SOFaa (80.5%), GC (64.6%) or OC (67.7%). Significant differences ($p<0.05$) were observed in morulae rates among culture with CR2aa (7.3%^{ab}), KSOMaa (3.8%^{a}), SOFaa (4.9%^{ab}), GC (7.2%^{b}) or OC (7.6%^{ab}). Refering to blastocyst (BL) and hatching (HA) rates no differences were observed among CR2aa, KSOMaa, SOFaa, GC or OC (BL = 46.6%; 34.7%; 31.3%; 26.1%; 24.7% and HA = 69.8%; 85.8%; 86.8%; 76.9%; 80.7%, respectively). In conclusion semi-defined medium and co-culture with primary cells under the same culture conditions have similar embryo development rates.

IN VITRO CULTURE OF BOVINE EMBRYOS IN SEMI-DEFINED MEDIA OR IN CO-CULTURE WITH CONTINOUS CELL LINE

Tavares, L.M.T.; Feitosa, W.B.; Caetano, H.V.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, visintin@usp.br

The culture system for in vitro production (IVP) an influence embryo development, metabolism and cleavage and pregnancy rates. The aim of this study was to evaluate the development of *in vitro* fertilized bovine embryos and cultured in vitro with chemically semi-defined medium CR2, KSOM and SOF added of aminoacids (aa) and 5%FCS or co-cultured in TCM199 medium with BRL or VERO cells with 5%FCS. Oocytes derived from slaughterhouse ovaries were in vitro matured for 24 hours in TCM199 + 10%FCS + HCG + FSH + 17ß estradiol. For in vitro fertilization, semen was thawed, centrifuged in Percoll gradient (45/90%) and diluted at final concentration of 1x106 spermatozoa/ml. The oocytes were fertilized (D0) in IVF medium (sp-TALP + PHE + heparin) under humidified atmosphere and 5%CO_2 in air at 38.7ºC for 18 hours. The presumable zygotes were cultured in 500µl of chemically semi-defined medium CR2aa (N= 427), KSOMaa (N= 422) or SOFaa (N= 421) or co-cultured in TCM199 with BRL (N= 360) or VERO (N= 364) under humidified atmosphere, at 38.7ºC and 5%CO_2 in air, during 10 consecutive days. Cleavage rate was evaluated at day 3, morulae and blastocyst at day 7 and hatching at day 10. The data were analyzed by ANOVA. No significant differences were observed ($p>0.05$) among CR2aa (83.4% and 3.2%), KSOMaa (73% and 0%), SOFaa (83.4% and 4%), BRL (76.1% and 9.5%) or VERO (71.4% and 3.1%) for cleavage and morulae rates, respectively. Differences ($p<0.05$) were observed in blastocyst rates among CR2aa (46.2%^{ab}), KSOMaa (55.8^{ab}), SOFaa (65%^{b}), BRL (33.6%^{a}) or VERO (40%^{ab}). No differences were observed in hatching blastocyst rates among CR2aa, KSOMaa, SOFaa, BRL or VERO (82.9%; 84.3%; 79.4%; 69.6% and 67.3%, respectively). In conclusion, KSOMaa showed better results with BRL cells and no differences were found between this and the others culture system.

EFFECT OF GLYCEROL AND ETHYLENE GLYCOL ON THE DEVELOPMENT OF *IN VITRO* PRODUCED BOVINE EMBRYOS

Nicacio, A.C.; Yamada, C.; Simões, R.; Mendes, C.M.; Caetano, H.V.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, anicacio@usp.br

The aim of this study was to evaluate the *in vitro* viability of *in vitro* produced bovine embryos after exposure to different cryoprotectant solutions. Bovine ovaries were collected at a slaughterhouse and oocytes matured, fertilized and cultured *in vitro*. The embryos were co-cultured with granulosa cells monolayer in SOF + 5%FCS and aminoacids. Expanded blastocysts between 7th and 9th days after insemination were exposed to treatment groups. Group EG were exposed to cryoprotectant solution of 10%Ethylene Glycol for 10 minutes or Group EG/Gly to solution of 10%EG for 10 minutes and then to solution of 20%EG and 20%Glycerol for 30 seconds. Cryoprotectants were diluted using PBS + 0.2% BSA + 0.3MSucrose solution and then PBS + 0.2%BSA solution, both for 3 minutes and hatching rate was evaluated after culture. As control, embryos not exposed to the cryoprotectant solution were cultured and evaluated for hatching. The hatching rates were 62.12% (41/66) for control group; 67.02% (63/94) for EG and 68.82% (64/93) for EG/Gly groups. Results were analyzed by Chi-Square Test. No differences were observed between control and EG groups as well as for EG and EG/Gly groups ($p>0.5$). But, differences were observed between control and EG/Gly groups ($p<0.5$). In conclusion, the cryoprotectants were not deleterious to the development of *in vitro* produced bovine embryos until hatching, but others studies must be done to evaluate the effects of this solutions during the cryopreservation.

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RESPIRATION BLOCKAGE OF *IN VITRO* BOVINE EMBRYOS

Mesquita, L.G.[1]; Cortezzi, S.S.[1]; Ripamonte, P.[1]; Chiaratti, M.R.[1]; Emanuelli, I.P.[1]; De Bem, T.H.C.[1]; Miranda, M.[1]; Porciuncula, P.M.[1]; Merighe, G.K.F.[1]; Leal, C.L.V.[1]; Meirelles, F.V.[1]

[1] Departamento de Ciências Básicas, Universidade de São Paulo, Pirassununga, São Paulo, Brazil, 13635-900. ligiagmesquita@yahoo.com.br

Mitochondria is a source of life (energy) under aerobic conditions; however, it also responsible for the programmed cell death signals. In the cellular respiratory process, there are drugs responsible for electrons transfer blockage of respiratory complexes. The potassium cyanide (KCN) acts in the fourth respiratory complex or oxidase cytocrome blocking electrons transfer to oxygen and consequent inhibition of protons dislocation to inner membrane and the ATP production. The mitochondrial membrane potential (?mm) is a key indicator of cellular viability and it is consequence of pumping ions across the inner membrane during the process of oxidative phosphorylation (OXPHOS). In the beginning of development, bovine embryos are independent of OXPHOS which increases during the cleavage process. Studies with bovine embryos submitted to oxidative stress and partial inhibition transports showed that ATP production by OXPHOS is not essential to embryo development. This work aimed to study the relation between ?mm with bovine embryos development submitted to treatment with blockage respiratory chain KCN. Oocytes were *collected from slaughtered cows* ovaries and submitted to maturation in bicarbonate-buffered TCM-199 supplemented Earles salts with 10% FCS, pyruvate, FSH, LH and 17ß estradiol for 22 hours. Following maturation, oocytes were fertilized in TALP medium supplemented with heparin, pyruvate, BSA and PHE solution for 18 hours and cultured in SOF medium supplemented with 10% FCS at 20% de O2. At 48 hours post insemination (hpi) 50% of the medium was replaced with 500?M, 1µM and KCN treatment absence. At 80 hpi the 8 cells embryos were submitted to JC-1 staining (1mg/mL) during 50 minutes to evaluate ?mm at fluorescence microscope. At 168 hpi blastocyst rate was evaluated in each treatment. In 1µM of KCN and control showed different ?mm (positive embryos to JC-1 staining) compared with 500?M de KCN (64, 70 e 80%, respectively); no significant differences were found in blastocysts development rate in the respective groups (15, 17 e 12%; $p<0.05$), using Chi-square according to GENMOD procedure in Statistical Analysis System program. These results suggest that the development may be independent of respiration or that part of embryos can be resistant to KCN dose i.e. embryos with ?mm at 48 hpi.

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EVALUATION OF THE MALE: FEMALE RATIO IN THE *IN VITRO* PRODUCTION OF BOVINE EMBRYOS

Stranieri, P.[1]; Accorsi, M.F.[1]; Bishop, W.[1]; Santos, R.G.[1]; Watanabe, M.R.[1]; Meirelles, F.D.P.[2]; Meirelles, F.V.[2]; Watanabe, Y.F.[1,2]

[1]Vitrogen® Pesquisa e Desenvolvimento em Biotecnologia da Reprodução, Cravinhos–SP, Brasil.,
[2]Depto de Ciências Básicas da FZEA-USP, Pirassununga-SP, Brasil.
patriciacg@vitrogenbr.com

The demand for animals of high genetic merit has been intense in bovine breeding, especially after the introduction of biotechniques applied for animal reproduction, such as embryo transfer, follicular aspiration and *in vitro* fertilization (IVF). The use of IVF, especially in zebu cattle, has favored the market for high genetic merit donors and consequently of recipients pregnant of female embryos. The aim of the present work was to verify if there is a variation regarding the male:female ratio in produced embryos *in vitro* at sexing at 60 days of pregnancy and if the bull factor is responsible for part of this variation. Embryos were produced *in vitro* according to the protocol described by Watanabe *et al* (Theriogenology, v.51 p.438, 1999). The oocytes were obtained by transvaginal follicular aspiration of several donors and matured, fertilized and cultured in vitro. After 7 days in culture, the embryos were transferred to previously synchronized recipients. Pregnancy detection was performed by ultrasound at days 30 and 35 of pregnancy, considering the visualization of the fetus and confirmation of viability by detection of a heart beat. Recipients diagnosed as pregnant were maintained separated, until submitted to a second ultrasound examination between days 70 and 80 to confirm pregnancy and determine the sex of the fetus. The presence of a genital tubercle near the umbilical cord led to the diagnosis of a male fetus. Data were analyzed by ANOVA and means compared by TUKEY-KRAMER HSD test. Sixty bulls were analyzed during a period of 13 months, producing 3,252 pregnancies at 30 days (39%), and from these 3,011 resulted in confirmed pregnancies by day 60 (36%), from which 1,286 were female (42.7%). Although there is not a statistical difference ($P>0.2$) among bulls in the frequency of conceived females, the percentage of pregnancies diagnosed female from evaluated bulls had a variation between $12.5 \pm 11.7\%$ to $84.0 \pm 14.8\%$. When analyzing the mean of females obtained in this work (42%) considering the expected mean (50%), there was a highly significant difference ($P=0.00000004$) for the diagnosis performed at 60 days of pregnancy. The causes for this deviation are still unclear and show the need for research in this area to develop methods that do not alter the sex ratio for commercial use.

BOVINE BLASTOCISTS PRODUCED AT CHEMICALLY DEFINED OR ORGANIC CULTURE SYSTEMS[1]

Soria, G.F.[2,3]; Perecin, F.[2]; Landim Jr., L.P.[2]; Resende, M.V.[2]; Meo, S.[2]; Basso, A.[2]; Paz, C.C.P.[4]; Vantini, R.[2]; Garcia, J.M.[2]; Esper, C.R.[2]

[2] Departamento de Reprodução Animal, FCAV/UNESP, Jaboticabal - SP, 14870-060, Brasil. [4] Agência Paulista de Tecnologias dos Agronegócios, Ribeirão Preto - SP, Brasil.
gabrielferreirasoria@yahoo.com.br

This study aimed to evaluate the effects of *in vitro* culture systems supplemented or not with different proteins sources at bovine embryos development. The chemically defined system was constituted by SOFaaci medium additioned with polyvinyl alcohol (0,3%) and Hepes (10mM) (SPH; n=1959). Organic groups were: 0,5% BSA (SB; n=1472), 5% FCS (SF; n=1384) and 2,5% FCS + 0,5% BSA (SF+SB; n=1263). Follicles between 2 and 8 mm of diameter were aspirated from abattoir ovaries and oocytes were maturated and fertilized (day 0) in air for 24 and 18 hours, respectively. Putative zygotes were denuded and randomly distributed at treatments and cultured at 38,5°C, saturated humidity and 5% O_2, 5% CO_2 and 90% N_2. Control group (CT; n=943) constituted by SF+SB was maintained in air at the same temperature and humidity conditions. At the third and seventh day of culture were verified in 18 replications, respectively, cleavage and blastocysts rates related to zygotes numbers. The means rates (mean ± S.E.M.) were evaluated by ANOVA and were compared using Tukey's test (SAS System). Cleavage rates of SPH (68.5±2.8%), SB (73.8±2.5%), SF (68.8±1.8%), SF+SB (72.8±2.7%) and CT (70.2±1.8%) systems didn't present any differences. Blastocysts rate of SPH group (6.2±2.0%) differed (P=0.0062) between CT (17.6±2.0%) but didn't for SB (10.8±1.9%), SF (12.2±2.4%) and SF+SB (10.7±3.1%); although, SB, SF, SF+SB and CT didn't show differences. It is possible to culture bovine blastocysts at low or high oxygen tensions at the presence or not of proteins sources.

[1]FAPESP 2003/07596-4. [3] CNPq scholarship – Animal Reproduction Graduating Program.

QUALITY OF BOVINE BLASTOCYSTS CULTURED AT THE PRESENCE OR NOT OF PROTEINS SOURCES[1]

Soria, G.F.[2,3]; Landim Jr., L.P.[2]; Resende, M.V.[2]; Perecin, F.[2]; Ferreira, C.R.[2]; Basso, A.[2]; Paz, C.C.P.[4]; Vantini, R.[2]; Garcia, J.M.[2]; Esper, C.R.[2]

[2] Departamento de Reprodução Animal, FCAV/UNESP, Jaboticabal - SP, 14870-060, Brasil. [4] Agência Paulista de Tecnologias dos Agronegócios, Ribeirão Preto - SP, Brasil.
gabrielferreirasoria@yahoo.com.br

Culture system influences embryo quality (Rizos, Biology of Reproduction, 68, 236-243) so this study aimed to assess bovine blastocysts quality cultured at SOF medium additioned with citrate, myoinositol and essential and non-essential amino acids (SOFaaci) and proteins sources or synthetics macromolecules. Blastocyst quality was evaluated by hatching rate considered a non-invasive technique (hatched blastocysts/blastocysts) (Van Soom, Reproduction Domestic Animal, 36, 29-35). Oocytes punctured from ovaries colected at abbatoir were maturated for 24 hours at TCM 199-Bicarbonate + 10% FCS, hormones, piruvate and antibiotic. Fertilization was performed at TALP + 0.6% BSA, heparin, PHE, piruvate and antibiotic. Presumptive zygotes were mechanically denuded and randomly allocated at culture systems groups: control (CT; n=178) constituted with 2.5% FCS + 0.5% BSA and atmosphere of 20% O_2; organics supplemented with 0,5% BSA (SB; n=110), 5% FCS (SF; n=109) and 2.5% FCS + 0.5% BSA (SF+SB; n=106) and chemically defined with 0,3% polyvinyl alcohol and 10mM of Hepes (SPH; n=72) were maintained at 5% O_2. Thirteen replicates for hatching rates were verified at day 9 for control and CT groups and day 10 for defined one. The means (mean ± S.E.M.) were evaluated by ANOVA and were compared using Tukey's test (SAS System). Hatching rates for SPH (4.7±3.5%) was lower than CT (49.6±7.3%) (P=0.0016) and SF (37.1±9.5%) (P=0.0421). SB (29.8±9.8%) and SF+SB (22.2±8.0%) groups didn't differed each other and also between CT and SPH. *In vitro* culture systems supplemented with proteins sources optimize blastocysts quality, independently of oxygen tension. Blastocysts cultured at chemically defined systems present low embryonary viability.

[1]FAPESP 2003/07596-4. [3] CNPq scholarship – Animal Reproduction Graduating Program.

DEVELOPMENT OF BOVINE BLASTOCYSTS AT CHEMICALLY DEFINED CULTURE MEDIA[1]

Soria, G.F.[2,3]; Basso, A.[2]; Landim, L.P.[2]; Resende, M.V.[2]; Saraiva, N.Z.[2]; Perecin, F.[2]; Paz, C.C.P.[4]; Vantini, R.[2]; Garcia, J.M.[2]; Esper, C.R.[2]

[2] Departamento de Reprodução Animal, FCAV/UNESP, Jaboticabal - SP, 14870-060, Brasil. [4] Agência Paulista de Tecnologias dos Agronegócios, Ribeirão Preto - SP, Brasil.
gabrielferreirasoria@yahoo.com.br

Bovine embryo production at chemically defined culture medium avoid the presence of pathogens and are useful to study *in vitro* blastocysts requirements (Lonergan, Reproduction of Domestic Animal, 38, 259-267). Defined culture media that were studied utilized SOFaaci which was supplemented with 0.3% polyvinyl alcohol + 10mM Hepes + insulin-transferrin-selenium (10μg/mL) (PHFC) or 2.5% serum-free synthetic supplement ("Knockout Serum Replacement") (KOUT). After IVM and IVF (day zero), denuded zygotes were allocated at treatment (11 replications): PHFC (n=860) and KOUT (n=793) and cultured in a mixture gaseous atmosphere (5% O_2, 5% CO_2 and 90% N_2). Control group (CONT; n=1037) was constituted by SOFaaci added with 2.5% FCS and 0.5% BSA and maintained in air. Feeding was performed at day 3 and also the cleavage rate. Blastocysts production and blastomeres numbers were evaluated at day 8. The means of cleavage and blastocysts rates and cells numbers (mean ± S.E.M.) were evaluated by ANOVA and were compared using Tukey's test (SAS System). PHFC showed lower cleavage rate (71.2±2.2%) and did not differed to CONT (75.0±3.0%), but differed to KOUT (80.1±1.5%) (P=0.0318). Blastocyst rate for CONT (14.1±2.3%) and KOUT (10.5±1.4%) didn't differed. Although, embryonary production at PHFC group (2.4±1.0%) differed between CONT (P=0,0001) and KOUT (P=0,0055). No differences were observed for cells numbers between CONT (118.9±6.4) and KOUT (123.0±5.0) and also between CONT and PHFC (98.5±6.4), but KOUT differed from PHFC. Serum-free synthetic supplement could be an alternative for *in vitro* production of bovine embryos at chemically defined culture medium.

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EFFECT OF THE ESTRADIOL AND THE PROGESTERONE IN THE DEVELOPMENT OF BOVINE EMBRYOS IN VITRO PRODUCED

Reis, A.N.[1]; Miranda, M.S.[2]; Silva, A.O.A.[1]; Sousa, A.J.O.[1]; Silva, L.K.X.[1]; Sousa, J.S.[1]; Ohashi, O.M.[2]

[1]Central de Biotecnologia de Reprodução Animal – UFPa, Castanhal-Pa, [2]Laboratório Fertilização in vitro – UFPa, Belém-Pa. areis@ufpa.br

This work had as objective to evaluate the bovine embryos development produced in vitro until blastocyst stage, co-cultured with bovine oviductal epithelial cells (BOECs) exposed or not to estradiol and progesterone (P_4). The ovaries were collected at a slaughterhouse and transported to the laboratory. Oocytes were selected for 24 h IVM, in TCM-199 with Bicarbonate supplemented with hormones, serum and antibiotics, in an atmosphere of 5% CO_2, in air at 38,5°C. The BOECs were cultured in TCM-199 with Bicarbonate supplement or not with estradiol (1 μg/ml) for 24 h, in the same conditions for IVM. Frozen-thawed semen was centrifuged in a Percoll gradients (45 and 90%) during 30 min at 200 x *g*, and later washed in TALP-Semen during 10 min and also 200 x *g*. The final concentration was of 10^6 sptz/ml in IVF medium (TALP supplemented with PHE, Heparin and BSA). The BOECs were washed after 24 h and added in the different groups in CR_2 medium, supplemented with or without P_4, according with the experimental group: Group 1 (CR_2 + P_4-100μg/ml and BOECs + Estradiol; n= 324); Group 2 (similar to the G1, without P_4; n= 315); Group 3 (CR_2 + P_4- 100 μg/ml, and BOECs without exposition to Estradiol; n= 325) and Group 4 (CR_2 + BOECs; n= 322). As statistical method it was used ANOVA ($P<0.05$) and the Tukey test. The percentage of cleaved zygotes (3º day) and blastocysts (7º day) was lower ($P<0.05$) in the group G3 (34.54% and 16.36%, respectively), that it only contained P_4 without exposition of the BOECs to Estradiol, as compared to the group G1 (53.49% and 29.26%), G2 (56.27% and 31.22%) and G4 (51.68% and 28.72%), probably for an inhibiting effect of the P_4 when used alone in the medium. We conclude that the association of the CEOBs with estradiol significantly did not increase the percentage of cleaved and blastocysts, however, when these cells were associates the P_4, these percentages decreased significantly.

USE OF SEQUENTIAL AND COMMERCIAL MEDIUM TO IN VITRO PRODUCTION OF BOVINE EMBRYOS: PRELIMINAR RESULTS

Zerbini, F.L.S.[1]; Fernandes, M.B.[1]; Elias, F.P.[1]; Vila, R.A.[1]; Galerani, M.A.V.[1]; Lôbo, R.B.[1]

[1]Departament of Genetics – FMRP/USP, Ribeirão Preto – SP, Av. Bandeirantes, 3900, 14040-030, Brasil. chico_zerbini@yahoo.com.br

Bovine embryos cultured in vitro have different metabolic requirements over the stages of development. Therefore specific culture medium for each stage is formulated, in opposite to traditional medium. As the metabolism of zygote differs from the metabolism of blastocyst, which is the best stage for embryo transfer (Gardner, Theriogenology, v. 49, p. 83–102), two or more culture medium are frequently used for all phases of in vitro development and increase embryo viability. In this study we used two sequential medium for embryo culture in vitro. 472 oocytes collected from 24 cows by Ovum Pick-up, were matured during 24 hours and then fertilized. 15 hours after the fertilization presumptive zygotes were transferred to Bovine Vitro Cleave medium (Cook Veterinary Products) +10% Fetal Calf Serum (FCS) in co-culture with cumulus cells, maintained in incubator at 38.5°C, 5% CO_2 and high humidity. At day 4 of culture, Bovine Vitro Cleave medium was removed and Bovine Vitro Blast medium (Cook Veterinary Products) + 10% FCS was added. After 163 hours, 180 blastocysts (38.1%) were selected and transferred into receptors, resulting in 81 pregnancies at 60th day (45%). The results have shown that commercial and sequential mediums can be utilized in routine protocols for in vitro bovine embryo production. Furthermore, the use of commercial competent medium for in vitro culture can decrease variations when medium is manufactured at the laboratory.

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IN VITRO BOVINE EMBRYO CULTURE IN PRESENCE OF GH, IGF-1 AND INSULIN

Ponchirolli, C.B.; Freitas, C.P.; Landim-Alvarenga, F.C.

Departamento de Reprodução Animal e Radiologia Veterinária- FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. carlaponchi@yahoo.com.br

To improve in vitro embryo development, many growth factors have been added to the culture media. The aim of this study was to investigate the influence of the addition of GH (growth hormone), IGF-1(insulin like growth factor*)* and insulin to *in vitro* bovine embryo culture media (IVC). Bovine slaughterhouse ovaries had there 2 to 8 mm follicles aspirated to obtain the oocytes. Five replicates were used. Selected ocytes were *in vitro* maturated at 38º C, in a humidified atmosphere of 5% CO_2 in air for 22-24h, in TCM 199 supplemented with 10% bovine foetal serum (BFS), 2,2 mg/ml sodium pyruvate, 1 mg/ml estradiol 17ß, 50 µg/ml LH, 5 µg/ml FSH and 75 µg/ml gentamicin. After maturation, oocytes were fertilized in Fert Talp for 12h, under the same condition. Presumptive zygotes were placed in 90 µl drops and cultured in five different groups in presence of granulosa cells (co-culture). Control group: HTF medium (*Human Tubal Fluid,* HTF®, Irvane) and BME (*Basal Medium Eagle,* BME®, Sigma), on 1:1 rate, supplemented with 10% BFS, 0,01% myo-inusitol and 75 µg/ml gentamicin (control medium); GH group: control medium + 100 ng/ml GH; IGF-1 medium: control medium + 10 ng/ml IGF-1; insulin group: control medium +1µg/ml insulin; GH+IGF-1+Insulin group: control medium + 100 ng/ml GH, 10 ng/ml IGF-1+ 1µg/ml insulin. For statistical analysis ANOVA was used.. The blastocyst production was improved when IGF-1 was added to culture media, comparing with the group that recieved all of the compounds (Control: 111 blastocystes/ 323 oocytes - 34%^{ab}; GH: 114/ 328- 35%^{ab}, IGF-1: 123/342- 36%^{a}; insulin: 113/329- 34%^{ab}; GH+IGF-1+insulin: 97/314- 30%^{b}) This results indicate that the addition of to many stimulatory factors has no benefit on embryo production. Moreover there were no differences between control, IGF-1, GH and Insulin groups. To best evaluation of the influence of these factors on embryo culture, embryonic quality assay must be performed.

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EFFECT OF TRANSPORT ON DEVELOPMENT OF VITRIFIED-WARMED BOVINE EMBRYOS

Polisseni, J.[1]; Ramos, A.A.[1,2]; Sá, W.F.[1]; Ferreira, A.M.[1]; Camargo, L.S.[1]; Folhadella, D.S.[3]; Serapião, R.V.[1]; Viana, J.H.M.; Nogueira, L.A.G.[3]

[1]Embrapa Gado de Leite - Juiz de Fora – MG, Brasil. [2]Empresa de Pesquisa Agropecuária de Minas Gerais – MG, Brasil. [3]Universidade Federal Fluminense – RJ, Brasil. jupol@powermail.com.br

A limitant factor for diffusion of vitrification technology is the absence of an adequate laboratory structure to warm procedure in farm The possibility to overcome this limitation is to warm the embryos before transport. The aim of this study was to evaluate the viability of *in vitro* produced bovine embryos submitted to different periods of transport (6h-12h), after warmed. Oocytes, obtained from ovaries collected from slaughterhouse were matured in TCM-199 medium (Gibco Labs, Grand Island, NY), with estrus cow serum (ECS) and FSH, in 5% CO_2 at 38,5ºC for 24 hours. After 18 hours of fertilization, with 2,0 x 10^6 spermatozoa/ml, the presumptive zygotes were co-cultured with *cumulus* cells in CR2aa with 10% of fetal calf serum (FCS), for 7 days, in the same conditions of maturation. Grade I and II blastocysts (according to IETS manual) were selected and vitrified after exposition to PBS solution with 5% FCS (HM), added with 10% ethylene glycol (EG) and 10% of dymetil sulfoxide (DMSO), for one minute, followed by HM solution with 20% EG and 20% DMSO, for 20 seconds. The embryos were loaded into open pulled straws (OPS) and plunged into liquid nitrogen. Warming was performed at 39ºC by embryo exposure to decreasing concentration of sucrose (0.25 and 0.15M), for five minutes in each step. The warmed embryos were distributed in three groups: G1: *in vitro* cultured after warmed (n=25); GII: embryo loaded in to straws and keep for 6 hours at 35ºC, before *in vitro* culture (n=29); and GIII: embryo loaded into straws and kept for 12 hours at 35ºC, before *in vitro* culture. The embryos were co-cultured with *cumulus* cells in TCM-199 micro droplets added with SFB. It was evaluated the re-expanded and hatching rates after 48 hours in culture. It were realized five repetitions for each group and the results were analyzed with Chi-square. Re-expanded rates among groups I, II and III were not different ($p>0.05$; 68%, 44.8% and 65.5%, respectively), as well as hatching rates (32%, 24% and 27.6%). The results show that it is possible to transport warmed *in vitro* produced embryo, for periods up to 12 hours. However, regardless the transport period, studies shoud be carried out to improve embryo development after warmed

IN VITRO PRODUCED BOVINE EMBRYOS EXPOSED TO ETHYLENE GLYCOL AND TREHALOSE

Mozzaquatro, F.D.[1,3]; Franco, V.C.L.[1,3]; Bunn, S.[2]; Santos, M.V.[3]; Silva, D.S.[1,3]; Pilla, L.F.C.[1,3]; Rubin, M.I.B.[3]; Silva, C.A.M.[3]

[1]Programa de Pós-Graduação em Medicina Veterinária/UFSM, Santa Maria/RS. [2]Centro de Ciências Agroveterinárias – CAV/UDESC, Lages-SC, 88.520-000, Brasil. [3]Embryolab – Departamento de Clínica de Grandes Animais - CCR/UFSM, Santa Maria-RS, 97105-900, Brasil.fmozzaquatro@yahoo.com.br

The aim of this study was to determine the toxicity of the EmCare® (ICPbio Limited – Auckland/ New Zealand) holding solution with the addition of ethylene glycol (EG) and trehalose (TREA) in the survival and development rates of bovine embryos *in vitro* produced to establish a protocol for ultra rapid cryopreservation. Aspirated *cumulus*-oocytes complexes from bovine ovaries collected at slaughterhouse were *in vitro* matured in TCM-199 containing 10% estrus cow serum for 24h using an incubator with 5% CO_2 at 39ºC and satured humidity. Fertilization was accomplished during 18h in FERT-TALP medium at the same temperature and gaseous atmosphere of the previous maturation phase. The zygotes were cultured for 8 days in SOFaaci medium with 5% estrus cow serum in incubator at 39ºC using plastic bags where a gaseous mixture was injected (5% CO_2 5% O_2 and 90% de N_2). Blastocysts and expanded blastocysts (n=90) of D7 and D8 (D0 = Fertilization day) were exposed to EmCare® holding solution with 3.0M ethylene glycol (EG), 3.0M EG + 0.1M, 0.3M or 0.5M trehalose, for 2 minutes at 35ºC. They were immediately cultured in 100µL SOFaaci droplets, under mineral oil, for 48h, incubated at 39ºC with 5% CO_2 in air. The viability rates of embryos in EmCare® holding solution + 3.0M EG (13/18; 72.2%), 3.0M EG + 0.1M TREA (10/18; 55.5%), 3.0M EG + 0.3M TREA (10/18; 55.5%) and 3.0M EG + 0.5M TREA (12/18; 66.6%) showed no differences (P>0.05) between the treatments and no-exposed embryos (14/18; 77.7%). The 3.0M ethylene glycol with trehalose in EmCare® holding allowed the blastocyst rehydration, re-expansion and hatching indicating it's low toxicity for *in vitro* produced bovine embryos.

Keywords: IVP, bovine, ethylene glycol, trehalose.

EFFICIENCY OF IMMATURE BOVINE OOCYTE VITRIFICATION

Yamada, C.; Nicacio, A.C.; Mendes, C.M.; Gonçalves, J.S.A.; Braga, F.A.; Assumpção, M.E.O.A; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, claudiay@usp.br

The aim of this study was to compare the efficiency of bovine oocyte vitrification using different solutions. In experiment I, the oocytes were only exposed to cryoprotectants in two steps, first in EG10 (PBS + 10% ethylene glycol (EG) + 10% fetal calf serum (FCS) + 0.25 M trehalose) for ten minutes and then in EG+D40 (PBS + 10%FCS + 20%EG + 20%DMSO + 0.5 M trehalose) or EG+D50 (PBS + 10%FCS + 25%EG + 25%DMSO + 0.5 M trehalose) or EG+GLY (PBS + 10%FCS + 25%EG + 25%Glycerol + 0.5 M trehalose) for 30s or 60s. In experiment II, the oocytes were exposed to freezing solutions for 30s, as described in experiment I, loaded into open pulled straws (OPS), plunged and stored in liquid nitrogen. In both experiments, the cryoprotectant dilution was performed in step wise: 1) PBS + 10%FCS + 10%EG + 1,0 M trehalose; 2) PBS + 10%FCS + 0,5 M trehalose and 3) PBS + 10%FCS for 3 minutes each step. The oocytes were matured for 24h in microdroplets of IVM medium, denuded, fixed with paraformaldehyde and triton, stained with Hoechst 33342 and evaluated under fluorescent microscopy. In each manipulation, oocytes were matured, fixed, stained and evaluated in regards to nuclear maturation as control group. The oocytes in metaphase II were considered mature. The results were analyzed by Tukey test, with significancy level of 1%. In experiment I, average maturation rates were not different among Control (81.3%), EG+D40 with 30s (74.2%) and 60s (71.9%), EG+D50 with 30s (73.1%) and EG+GLY with 30s (74.5%), but EG+D50 and EG+GLY with 60s exposure showed lower maturation rates (56.2% and 54.3%). In experiment II, maturation rates of vitrified groups were lower than in control group (75.9%), but EG+D50 group (29.2%) was higher than EG+D40 and EG+GLY groups (11.7% e 4.3%). In conclusion, in this work, the most efficient protocol for immature bovine oocyte vitrification was EG+D50 with 30s of exposure, but it needs to be improved.

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EFFECT OF MATURATION TIME ON CATTLE OOCYTE VITRIFICATION

Magnusson, V.; Yamada, C.; Tavares, L.M.T.; Nicacio, A.C.; Assumpção, M.E.O.A; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, visintin@usp.br The aim of this work was to compare the efficiency of bovine oocyte vitrification after 0, 12 or 18 hours of maturation. Oocytes were collected from slaughterhouse ovaries and distributed in the groups: Control (matured for 22 hours), G-0 (vitrified without maturation), G-12 (matured for 12 hours and vitrified) and G-18 (matured for 18 hours and vitrified). For vitrification, the oocytes were exposed for 15 minutes in TCM199/Hepes + 20% of fetal calf serum (FCS) + 3%ethylene glycol (EG) + 5µgCitochalasin D/ml and exposed for 25 seconds in vitrification solution TCM199/Hepes + 20%FCS + 40%EG + 5µgCitochalasin D/ml. The oocytes were loaded in open pulled straws (OPS), plunged and stored in liquid nitrogen. The oocytes of groups G-0, G-12 and G-18 were warmed, washed twice for 5 minutes in TCM199/Hepes medium + 20%FCS and once in IVM medium and matured in microdrops of IVM medium under mineral oil for 22, 10 or 4 hours, respectively GO, G12 and G18, to complete 22 hours of maturation. After maturation, the oocytes were fertilized (DO) and the presumptive zygotes were co-cultured in microdrops of TCM199 with granulosa cell monolayer under 39ºC, 5% of CO_2 in air and humidified atmosphere. The cleavage, blastocyst and hatched blastocyst rates were evaluated on days 3, 9 and 12 of culture, respectively. The results were analyzed by Chi-square test, with significance level of 5%. The cleavage, blastocyst and hatched blastocyst rates were, respectively, Control (79.2; 51.4 and 41.4%); G-0 (26.5; 15.3 and 0%); G-12 (15.3; 7.1 and 0%) and G-18 (7.3; 4.2 and 0%). The vitrified groups showed cleavage, blastocyst and hatched blastocyst rates lower than control group. The cleavage and blastocyst rates of G-0 were higher than G-18, but G-12 rates were equal compared to G-0 and G-18. In conclusion, the vitrified groups showed lower embryo development results than control group, but oocytes without maturation appear to be more resistant to vitrification.

VIABILITY OF VITRIFIED BOVINE OOCYTES WITH TREHALOSE

Hastenreiter, G.P.[1]; Dias, A.J.B.[1]; Matos, L.F.[1]; Carvalho, C.S.P.[1]

[1]Laboratory of Animal Genetic Improvement /LMGA/ CCTA/ UENF,
Campos dos Goytacazes-RJ, 28013-600, Brazil. hannatomodati@yahoo.com.br

Trehalose has been used in some protocols of cryopreservation of tissue, gametes and embryos, with promising results, reducing of the cryopreservation effects. Because of its high molecular weight and biochemistry characteristics, this sugar is unable to cross the plasma membrane of the oocytes. The aim of this work was to evaluate the cryoprotective capacity of intracellular trehalose, on the viability of vitrified bovine oocytes. Oocytes (*n*=105) collected from slaughterhouse-derived ovaries were matured *in vitro* for 22h and later distributed randomly in the following treatments: oocytes evaluated after the MIV (T1); oocytes only had been perforated by the microinjection pipette (T2); oocytes had been microinjected with solution of trehalose 0.8M, until an intracellular concentration of 0,15 M (Eroglu, et. al, 2002) (T3); oocytes had the same procedure from T3, followed of load in OPS and vitrification (T4); vitrified in solution of DMSO and Ethylene glycol (EG), according to Vajta *et al.* (1998) (T5). The microinjection of trehalose was carried through with microinjector Transjector 5246 (Eppendorf), connected an inverted microscope Axiovert (Zeiss). The warming was carried through immerging the straws into solution contends sucrose. After the manipulations the oocytes had been kept by four hours in culture *in vitro* conditions, previously to the viability evaluations. The cellular viability was esteemed by the degree of return of the oocytes to its isotonic volume after 4 hours of culture, cellular disruption and marking with propidium iodide (PI). Oocytes showing red fluorescence (PI staining) were considered nonviable. Oocytes of the T2 and T3 treatments had presented 100% of return and no cellular disruption was observed. When submitted to the marking with PI, oocytes of both treatments they had respectively presented cellular viability of 80% and 60% rates. Ocytes of T4, had presented 63% of re-expansion and 29% of cellular disruption, however only 23% of the oocytes had met complete, not presenting marking with PI. Superior result was gotten with oocytes of T5. In this treatment 89% of the oocytes had returned, 10.5% had disrupted and only 22% had marked with PI. Our results corroborate the described information already in literature on the cryprotector potential of trehalose, however have necessity of if evaluating the more efficient effect of different concentrations and procedures for its use in the freezing of bovine ovócitos.

EFFECT OF ADITION OF SUCROSE TO THE VITRIFICATION MEDIUM OF *IN VITRO* PRODUCED ZEBU EMBRYOS

Varago, F.C.[1]; Lagares, M.A.[1]; Alvim, M.T.T.[2]; Saliba, W.P.[3]; Vasconcelos, A.B.[1]; Elias, N.Q.[2]; Stahlberg, R.[3]; Oliveira, C.H.[1]

[1] Departamento de Clínica e Cirurgia Veterinária - UFMG/Belo Horizonte – MG, [2]Cenatte Embriões - Pedro Leopoldo – MG, [3] Faculdade de Veterinária – PUC/Betim-MG, Brasil
fafavarago@terra.com.br

The national *in vitro* production of bovine embryo has a prominent position in the world scenery. These rates are mainly due to embryo production of zebu races. Although cryopreservation of this genetic material has been made, the embryo viability pos warming is not satisfactory. The aim of this study was to evaluate the effect of sucrose addition to the vitrification medium of *in vitro* produced zebu embryo. Oocytes were recovered by OPU from zebu cows. The oocytes were matured in vitro for 24 hours (5% of CO_2, in air and saturate humidity). For fertilization (D0), semen from a Nelore bull was used. On the seventh day of culture (5% of CO_2, 5% of O_2, 90% of N_2 in saturate humidity), 64 blastocysts classified as excellent quality were selected, measured and vitrified. The blastocyst production rate was 33% Two vitrification treatments were tested: T1) 25% of ethylene glycol + 25% of dimethyl sufoxide + 50% of holding medium (n=30) and T2) 20% of ethylene glycol + 20% of dimethyl sufoxide + 10% of sucrose solution + 50% of holding medium (n=34), and the addition of cryprotectors was made in four steps. After 40 seconds of exposition to the vitrification solutions, the embryos were loaded in “Open Pulled Straw” (“OPS”) and submerged into liquid nitrogen. The embryos were warmed in air for 3 seconds, re-hydrated in decreasing concentrations of sucrose, and culture for 48 hours. After 24 hours of culture, the embryos were measured again and re-expansion rates evaluated (30% and 44.1%). After 48 hours hatching rate were evaluated (13.3% and 26.7%). The sucrose addition to the vitrification medium did not improve the re-expansion and hatching rates after warming when compared to treatment 1 ($p>0.05$). Furthermore, due to the fewdata in the literature concerning cryopreservation of *in vitro* produced zebu embryos, the results are encourages. Nevertheless, more studies are necessary to elevated the viability of *in vitro* produced zebu embryos.

PREGNANCY OF FRESH AND VITRIFIED BUFFALO EMBRYOS PRODUCED *IN VITRO*

Sá Filho, M.F.[1]; Carvalho, N.A.T.[1]; Gimenes, L.U.[1]; Torres-Júnior, J.R.[1]; Ferreira, C.R.[2]; Perecin, F.[2]; Perini, A.P.[2]; Tetzner, T.A.D.[2]; Vantini, R.[2]; Soria, G.F.[2]; Garcia, J.M.[2]; Tonhati, H.[2]; Gasparrini, B.[3]; Baruselli, P.S.[1]

[1]Departamento de Reprodução. Animal, FMVZ-USP, São Paulo-SP,
[2] UNESP, Jaboticabal-SP, [3] Universtà degli Studi Frederico II, Napoli, Itália.
manoelsa@usp e barusell@usp.br

In the buffalo species ovum pick-up (OPU) programs and vitro embryo production (IVP) present difficulties, as poor number and quality of oocytes recovered by session and low *in vitro* fertilization rates. Aiming improve the efficiency of OPU in this species, ten buffalo heifers (*Bubalus bubalis*) were selected according to cyclicity, ovarian diameter (≥ 2cm) and number of follicles (≥ 8 follicles per ovarian). The females were submitted to five OPU sessions (twice a week), using 100 Falco Vet (Pie Medical) ultrasound, micro-convex probe and 19G needle diameter, with 60-65 mmHg aspiration vacuum. A total of 257 oocytes were recovered of 419 follicles punctured (61.3% recovery rate). The viable oocytes (n=247) were submitted to maturation (TCM199 + 10% bovine calf serum (BCS) + hormones + 0,3mM cystine + 50μM cysteamine), to fertilization (Talp-Fert) and to culture (SOF + 5% BCS) *in vitro*. The cleavage and embryo production rates were 45.3% (112/247) and 23.1% (57/247), respectively. The embryos produced in the first four sessions were vitrified, while that obtained in the last session (n=7) were destined to the fresh transference. The embryos were fixed-time transferred (FTET) to presynchronized recipients (GnRH-7days-PGF$_{2\alpha}$-2days-GnRH-7days-TETF+GnRH). The percentage of the transferred/treated were 80% (32/40) and the pregnancy rate were 14,3% (1/7) fresh and 8,0% (2/25) vitrified embryos. The conquest of the first America's pregnancies of fresh and vitrified buffalo embryos produced *in vitro*, demonstrated the possibility of establishment of viable OPU-IVP program in buffalo species. Further studies should be realized to evaluate the best aspiration interval and the development of strategies to improve *in vitro* fertilization rates in buffalo species.

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EFFECT Of TIME BETWEEN THAWING AND FIXED TIME EMBRYO TRANSFER OF ETHYLENE GLYCOL FREEZED EMBRYOS

Peres, L.C.[1,3]; Pincinato, D.[13]; Borges, L.F.K.[2]; Maraña Peña, D.[1,3]; Tribulo, R.[3]; Cutaia, L.[3]; Bó, G.A.[1,3]

[1]Universidad Nacional de Córdoba, Argentina, [2]Universidade Federal de Santa María, Brasil, [3]Instituto de Reproducción Animal Córdoba (IRAC), Jerónimo Luis de Cabrera 106, X5000GVD, Córdoba, Argentina. gabrielbo@iracbiogen.com.ar

The objective of this work was to evaluate the effect of the time (seconds) between thawing of ethylene glycol 1,5 M freeze embryos and the fixed time embryo transfer (FTET) on the pregnancy rates. Non-lactating multiparous crossbred Zebu, dry, cyclical cows (n=216) with body condition score between 3 and 4 (1 to 5 scale) were synchronized with a FTET protocol. The cows received a Crestar ear implant (Crestar, Intervet, Brazil) or a DIB device (Syntex, Argentina), plus 2 mg of estradiol benzoate (EB; Syntex) on Day 0 and 400 UI of eCG (Folligon 5000, Intervet or Novormon 5000, Syntex) more 150 µg de D (+) cloprostenol i.m (Preloban, Intervet or Ciclase Syntex) on Day 5. On Day 8, the implants and devices were removed and 24 h later the cows were injected with 1 mg of EB (Syntex). On Day 16, all the cows were ultrasound and those with a CL > 256 mm were FTET on Day 17. The transferences were classified in 3 groups: Group 1 time between thawing and transfer less than 180 seconds; Group 2: between 181 to 360 seconds and Group 3: more than 361 seconds (between 361 to 805 seconds). After 28 days from embryo transfer, the cows were ultrasound to determine pregnancy rate. The pregnancy rate were evaluated by logistic regression (Infostat, Argentina). There was not significant difference between groups (Group 1: 47/74 63.51%; Group 2: 115/204 56.37%; Group 3: 8/16 50%). However it would be more embryo transfers to evaluated if the time between thawing and ET have effect on pregnancy rates in crossbred Zebu recipient.

THAWING EFFICIENCY OF OVINE EMBRYOS OF DORPER BREED

Andrade, J.C.O.[1]; Sousa, B.P.A.[1]; Guido, S.I.[1]

[1] Av. Caxangá, 205, Sl 605, Madalena, Recife-PE, 50.610-230, Brasil.

The objective of this work was to evaluate the efficiency of ovine embryos thawing of DORPER breed on the fertility. The embryos (n=441), imported from South Africa, cryopreserved in EGLY at 1.5 M and packaged one to four in straws of 0.25 mL, were withdrawn from N and exposure to the air for 5" and than deepen in steamer for 20" at a temperature varying from 28 to 37 ºC. The embryos were submitted to for different protocols of thawing composed of the following solutions: A – BSA; B – sacarose at 0.5 M; C – EGLY at 0.75 M and D – made up of two solutions: D - sacarose at 0.5 M + EGLY at 0.75 M and D - sacarose at 0.5 M, the embryos were kept in each solution for 5' and following that the embryos received two bath in BSA solution., being evaluated according to the development (Mo, Bi, Bl e Bx) and quality (Grade I, II, III e IV). It was transferred by semi laparoscopy one embryo by recipient synchronized with protocol to TETF. The in ovulation took place into the horn ipsilateral to the ovary that showed a greater quantity and/or quality of CL. The data were analyzed by the Pearson χ^2 test. It was observed the following pregnancy rates: 1- regarding to protocols were 63.0% (63/100) in A, 47.1% (24/51) in B, 42.9% (27/63) in C and 51.1% (116/227) in D. 2- according to temperature were 55.4% (31/56) in the interval from 28 to 31 ºC, 49.5% (96/194) from 31.1 to 34 ºC and 53.9 (103/191) from 34.1 at 37 ºC. 3- Acording to the stage of development in origin were 51.9% (40/77) Mo, 60.3% (88/146) Bi, 46.9% (84/179) Bl and 46.2% (18/39) Bx. 4 – Concerning to the stage of embryo development after thawing were of 55.2% (181/328) Mo, 50.9% (27/53) Bi, 38.0% (19/50) Bl and 30.0% (3/10) Bx. 5 – Referring to embryo quality were 55.9% (90/161) GI, 59.8% (116/194) GII, 29.2% (19/65) GIII and 23.8% (5/21) GIV. 6 – With relation to ovulation rates of recipient were 48.5% (110/227) showing one CL, 56.1% (97/173) two CL and 56.1% (23/41) three or more CL. It is concluded that the conception rate of 52.2% (230/441) among the analyzed variants was influenced ($p<0.05$) regarding only to the grade of the embryo quality in the post thawing.

THE USE OF FLUORESCENT PROBES TO EVALUATE EQUINE CULTURED FIBROBLASTIC OBTAINED FROM SKIN FRAGMENTS

Lima Neto, J.F.; Fernandes, C.B.; Landim-Alvarenga, F.C.

Department Animal Reproduction - FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. joaoferreiralima@yahoo.com.br

The analyses of *in vitro* cultured cells is important for the determination of *in situ* viability, morphologic quality, and quantitative evaluation. In this study, fluorescent probes were utilized to analyze the viability of equine fibroblast cultured *in vitro*. Six samples of skin fragments, with about $1cm^2$ of diameter, were obtained from six equine females. The fragments were divided on pieces with about $1mm^3$ with the use of a surgical blade. The material was washed 5 times in a centrifuge tube, with Calcium and Magnesium free PBS solution, pH 7.4, using a vortex. The PBS was replaced on every wash. For culture the media was homogenized and distributed in two culture bottles ($25cm^2$). The bottom of the bottles was humidifying with 1ml of SFB and the 10 fragments of skin deposited. The bottles were then transferred to a vertical position and 5 ml of DMEM + 10% SFB, 100UI/ml Penicillin, 100μg/ml Streptomycin and 3,0 μg/ml Anfotericina B were added. After 40 minutes incubation on 5% CO_2 in air, the bottles were moved to a horizontal position allowing the culture media to get in touch with the tissue fragments. The cultured was performed for 7 days in 5% CO_2 in air until the beginning of cell growth. The complete culture media was replaced weekly and with 70% of confluence the tripsinization (ATV Vernese, Adolfo Lutz Institute) and the first passage were performed. The procedure was repeated for the second passage. Samples were colleted during the cell suspension for the second passage, and at the end of it. A sample of each bottle was collected and suspended in a 30mm Petri dish (Corning®) containing 1mL FBS. The dish was then cultured for 24 hours in 5% CO_2 in air before staining. It was compared the use of Propidium Iodite (PI - Sigma P4170 - 50μg/mL) associated with Hoescht 33342 (Sigma B2261 - 25 μg/mL) or with Acridine Orange (USB 10390 - 100μg/mL). The fluorescent probes were added to the bottom of each Petri dish, one at the time, followed by a 5 min. incubation time. A bath with PBS was performed between probes. An inverted fluorescent microscope (Leica DMIRB) was utilized. When a UV filter (PB 350 – 450 nm) was used viable nuclei stained in blue by the Hoescht 33342 and dead cell nuclei stained in pink by the PI. When the blue filter (PB 450 – 490 nm) was used normal cell nuclei stained in green by the Acridine Orange and dead cell nuclei stained in red by PI. Using this last protocol was also possible to analyze the morphology of the nuclei; were cells wit fragmented nuclei were considered apoptotic. It was concluded that both protocols were useful to analyze the viability of equine fibroblast in culture. However the dual stain PI/Acridine Orange offers a more clearly fluorescent pattern with the possibility to analyze nuclear morphology.

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PRIMARY CULTURE STANDARDIZATION OF EQUINE FIBROBLAST FROM SKIN FRAGMENTS

Fernandes, C.B.; Lima Neto, J.F.; Landim-Alvarenga, F.C.

Department Animal Reproduction – FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil, fernandescb@yahoo.com.br

The culture of cells and tissue has been extensively studied in the last decades because its application in several fields of research. However, for the equine species some special factors seem to have an influence, on the primary fibroblast culture obtained from skin fragments. The object of this study was to develop an efficient technique for primary culture of equine fibroblasts on the conditions of our laboratory, developing an equine cell bank for use on further researches. Six samples of skin fragments, with about $1cm^2$ of diameter, were obtained from six equine females (property of Animal Reproduction Department FMVZ- UNESP, Botucatu). The fragments were processed though two protocols: a hemi fragment was chopped on small parts with the use of a curve ophthalmic scissor, or a hemi fragment was divided on pieces with about $1mm^3$ with the use of a surgical blade. The material of both groups was washed 5 times in a centrifuge tube, with Calcium and Magnesium free PBS solution, pH 7.4, using a vortex. The PBS was replaced on every wash. The culture was performed using two different protocols. To the E1 group 10 ml of DMEM culture media (Gibco 11995-065) were used, supplemented with 10% SFB, 100UI/ml Penicillin, 100μg/ml Streptomycin and 3,0 μg/ml Anfotericin B. The media was homogenized and distributed in two culture bottles ($25cm^2$). To the E2 group the bottom of two culture bottles were humidify with 1ml of SFB and the fragments of skin where deposited. The bottles were then transfered to a vertical position and 5 ml of DMEM + 10% SFB, 100UI/ml Penicillin, 100μg/ml Streptomycin and 3,0 μg/ml Anfotericina B were added. After 40 minutes incubation on 5% CO_2 in air, the bottles were moved to a horizontal position allowing the culture media to get in touch with the tissue fragments. Both groups were cultured for 7 days in 5% CO_2 in air until the beginning of cell growth. The complete culture media was replaced weekly and with 70% of confluence the tripsinization (ATV Vernese, Adolfo Lutz Institute) and the first passage were performed. The procedure was repeated for the second passage. For each new passage 20% SFB was added to the culture media. Two passages were performed before freezing. In the conditions of this experiment it was observed that the tissue fragmentation with the use of a surgical blade resulted in lower bacterial contamination when compared with the use of scissors. Moreover the culture technique used on E2 group leads to a more uniform cell growth when compared to E1 group. The cells in culture presented fibroblast-like morphology since the beginning of culture and the proportion of growth in the E2 group did not change since their isolation, indicating a high cloning efficiency.

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ANALYSIS OF THE CELL CYCLE OF GRANULOSA CELL CULTURE DERIVATED OF ASPIRATED FOLLICLES OF NELORE CATTLE FOR NUCLEAR REPROGRAMMING

Caetano, H.V.A.; Schumacher, R.I.; Goissis, M.D.; Oliveira, V.P.; Feitosa, W.B.; Nicacio, A.C.; Gonçalves, J.S.A.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
helocae@yahoo.com.br

Different cellular types have been used for animal cloning. These nuclear donors, before being introduced in oocyte cytoplasm, are induced to the G0 phase by reduction of the concentration of serum in the culture medium. The aim of the present work was to evaluate the cell cycle of the granulosa cells obtained from ultra-sound aspirated follicles of Nelore heifers. The lineages remained in standard culture, with 10% of serum (control) and the ones in semi - confluence were treated for 24, 48, 72, 96, 120 and 144 hours in medium with 0,5% of serum. The analysis of the cell cycle showed that in control were observed 84.7% of cells in phase G0+G1; 2.3% in phase S; 7.3% in phase G2+M and 2.0% of dead cells. When these lineages were submitted to 24h of serum starvation, it was observed 84.7% of cells in phase G0+G1; 2.3% in phase S; 6.0% in phase G2+M and 4.6% of dead cells. After 48h of starvation, it was observed 67.6% of cells in phase G0+G1; 1.4% in phase S; 5.0% in phase G2+M and 21.5% of dead cells. After 72h of starvation, it was observed 63.1% of cells in phase G0+G1; 1.4% in phase S; 3.5% in phase G2+M and 25.95% of dead cells. After 96h of starvation, it was observed 74.67% of cells in phase G0+G1; 8.45% in phase S; 6.7% in phase G2+M and 7.5% of dead cells. After 120h of starvation, it was observed 64.4% of cells in phase G0+G1; 2.9% in phase S; 1.2% in phase G2+M and 28.7% of dead cells. After 144h of starvation, it was observed 63.1% of cells in phase G0+G1; 1.4% in phase S; 3.5% in phase G2+M and 25.9% of dead cells. The granulosa cells lineages had high percentage of cells in G0+G1 in all analyzed periods. There was an increase on the percentage of dead cells in cultures that remained for 72 to 96h and 144h on starvation. The cells that had survived, increased the percentage of cells in phase G2-M in cultures of 120h on starvation. There was a new increase of the percentage of dead cells in culture that remained for 120h on starvation. The granulosa cells showed high percentage in phase G0+G1 on control and starvation groups, therefore they could be used in any period for nuclear transfer.

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ANALYSIS OF THE CELL CYCLE OF ADULT AND FETAL FIBROBLASTS AS SOURCES FOR NUCLEAR TRANSFER IN NELORE CATTLE

Caetano, H.V.A.; Schumacher, R.I.; Marques, M.G.; Yamada, C.; Simões, R.; Mendes, C.M.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
helocae@yahoo.com.br

The success of somatic nuclear transfer depends on the cell cycle stage of the donor nucleus and the recipient cytoplasm. The objective of this work was to establish lineages derived fibroblasts of adult and fetal bovine. It was analyzed the cell cycle using flow cytometry (FACScalibur) of control culture (10% of FCS) and in cultures that remained for 24, 48, 72, 96, 120 and 144h in serum starvation (0.5% FCS). Parametric analysis were tested by ANOVA e $P < 0.05$ were considered significant. The analysis of the cycles of adult and fetal fibroblasts that remained with control medium showed that cells behave in similar way. There was in the lineages of the culture standard analyzed 71.1% and 80.7% they were in phase G0+G1; 2.7% and 3.9% in phase S and 18.8% and 10.8% in phase G2+M, respectively for adults and fetal fibroblasts. In fetal lineages that remained for 24h and 120h in serum starvation, lower percentages of cells in phase G0+G1 were observed; (2.5% - 24h and 1.54 - 120h). In these periods there is an increase in the percentage of dead cells (92.3% - 24h and 95.3% - 120h). In other starvation periods the percentage of cells in phase G0+G1 was not increased in relation to control; (58.4%; 77.5%; 69.6%; 82.5% respectively 48h; 72h, 96h, 144h). The adult lineages showed at 48h and 72h of starvation a lower percentage of cells in G0+G1 than control; (47.0% - 48h and 50.4% - 72h). In other starvation periods no difference in relation to the control was observed; (78.2%; 78.9%; 71.4%; 76.4; respectively 24h, 96h, 120h, 144h). It was concluded that for these cell lineages the starvation periods did not increase the percentage of cells in G0-G1.

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CELL CYCLE ANALYSIS OF ADULT FIBROBLAST FOR NUCLEAR REPROGRAMMING IN EQUINE

Caetano, H.V.A.; Schumacher, R.I.; Nascimento, A.B.; Martins, L.F.; Milazzotto, M.P.; Coutinho, A.R.S.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
helocae@yahoo.com.br

Normal development of cloned embryos depends on G0 or G1 cell cycle stage on nuclear transfer. The aim of this present work was to evaluate the cell cycle of equine fibroblast cultures submitted to serum starvation (0,5% FBS) for 24, 48, 72, 96, 120 and 144 hours. Primary culture of fibroblasts derived from oral tissue of an adult stallion was established. Cell cycle was determined by flow cytometry using propidium iodide as quantitative DNA marker. Analysis showed on the control culture (5% FBS) 66.5% of cells in G0+G1 stage; 12.3% in S; 17.1% in G2+M and 1.4% of dead cells. After 24 hours of serum starvation cultures 17.8% of cells in G0+G1 stage were observed; 1.4% in S; 0.4% in G2+M and 38.4% of dead cells. After 48 hours of serum starvation 9.88% of cells in G0+G1 stage were observed; 1.2% in S; 0.6% in G2+M and 38.7 of dead cells. After 72 hours of serum starvation 16.1% of cells were in G0+G1 stage; 3.1% in S; 1.2% in G2+M and 20.8% were dead cells. After 96 hours of serum starvation 17.5% in G0+G1 stage were observed; 3.9% in S; 1.3% in G2+M and 15.4% of dead cells. After 120 hours of serum starvation 4.5% of cells were on G0+G1 stage; 1.0% in S; 0.3% in G2+M and 67.1% were dead cells. After 144 hours of serum starvation 85.2% in G0+G1 stage were observed; 4.1% in S; 1.4% in G2+M and 6.2% of dead cells. The proportion of cells in S and in G2+M stage remained constant on analyzed periods, but high rates of dead cells were observed in all treatments, except for 144 h. It was concluded that equine fibroblast lineages are very sensible to decrease of serum concentration in media. Fibroblasts that survived serum starvation and duplicated, showed high proportion of cells in G2+M after 120 hours. Serum starvation for 144 hours is the best period to obtain cells in G0+G stage for nuclear transfer.

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CHARACTERIZATION AND CELLULAR CYCLE ANALYSIS OF ADULT DOG FIBROBLAST (Great Dane)

Caetano, H.V.A.; Schumacher, R.I.; Yamada, C.; Gonçalves, J.S.A.; Goissis M.D.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil, helocae@yahoo.com.br

Fibroblasts of adult animals can be used as nuclear donor on nuclear transfer and as models for studies of genomic activation, nuclear reprogramming, cell cycle synchronization and nucleus-cytoplasm interactions. An important factor for success of nuclear reprogramming in cloned embryos from somatic cells is the coordination of the cell cycle phase. The aim of this work was to establish primary culture of adult dog fibroblasts. In addition, this research investigated the distribution of cell cycle phases in control (5% FBS) or serum starvation (0.5% FBS) for 24, 48, 72, 96, 120 and 144 hours cultures. Canine fibroblasts were morphological and ultra-structural characterized. The lineages were characterized by indirect imunofluorescence, using the anti-vimentin antibody that is a protein marker of intermediate filament. After the third passage, all the cells presented positive marking. The cell cycle was determined by flow cytometry (FACS), using propidium iodide as quantitative DNA marker. The analysis demonstrated that in control culture 48.5% of the cells were in phase G0+G1; 1.2% in phase S; 40.4% in phase G2+M and 2.8% were dead cells. In culture after 24h of serum starvation 50.9% of the cells were in phase G0+G1; 1.4% in phase S; 37.7% in phase G2+M and 3.2% were dead cells. After 48h of starvation it was observed 56.5% of the cells in G0+G1; 1.4% in S; 30.5% in G2+M and 4.2% dead cells. After 72 hours of serum starvation 56.5% of the cells were in G0+G1; 1.4% in S; 31.4% in G2+M and 3.1% dead. After 96h of starvation 1.2% of the cells were in G0+G1; 0.6% were in S; 0.4% were in G2+M and 88.0% were dead. After 120h of starvation it was observed 56.7% of the cells in G0+G1; 1.2% in S; 34.4% in G2+M and 1.7% dead. After 144h of starvation 48.2% of the cells were in G0+G1; 2.0% in S; 25.5% in G2+M and 7.3% were dead cells. The analysis of these data showed that the percentage of cells in phase S remained constant in all analyzed periods. Also, there was no variation in the percentages of cells in phase G0+G1 and G2+M, except for 96h of serum starvation. In this period was seen a high percentage of dead cells and an abrupt decrease of percentage of cells in phase G0+G1 and G2+M. This ratio increases with 120h of serum starvation. By these results, it is concluded that serum starvation for 72h is the best period for nuclear transfer being cells in G0+G1 phase of the cell cycle.

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EFFECTS OF FETAL CALF SERUM STARVATION OR CICLOHEXEMIDE TREATMENT ON PORCINE FETAL FIBROBLASTS

Goissis, M.D.; Caetano, H.V.A.; Marques, M.G.; Nascimento, A.B.; Oliveira, V.P.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
mgoissis@yahoo.com.br

The success of nuclear transfer depends on many factors, as the donor cell cycle stage. It is described that donor cells on G1 or G0 stages allow genetic *loci* translocation, contributing for epigenetic reprogramming. Donor cells on G0 or G1 stages are also important for ploidy maintenance when using non pre-activated oocytes with high MPF levels. To improve efficiency of nuclear transfer, synchronization of donor cells is an important issue. Many methods of synchronization are described, including protocols using drugs or fetal calf serum (FCS) starvation. The aim of this study was to observe the effects of FCS starvation or ciclohexemide (CHX) treatment on porcine slaughterhouse fetal fibroblasts. A cell lineage was obtained from approximately 45 days fetus and it was cultured until fifth passage with DMEM media containing 10% FCS when was changed to treatments media. Treatments were 0.5% FCS for 1 to 6 days, 10µg/ml CHX with 10% FCS for 12, 24 and 48 hours or 10% FCS as control. Cells were stained with hematoxilin-eosin (HE) for observation on an epifluorescence microscope and also submitted to viability tests with trypan blue, which stains dead cells. Control cells had homogeneous cytoplasm, delimitated nucleus with evidenced nucleolus, visible cytoplasmic limits and prolongations. FCS starvation for 1, 2 and 3 days did not show any cell alterations in relation to control. Starvation for 4 days led to few alterations of cytoplasmic contour, indicating cellular injury. Starvation for 5 days showed increased eosinophilic staining, also indicating cellular injury. After 6 days starvation cells showed hetrogeneus cytoplasm, disarranged cytoplasmic filaments, morphologic deteriorations, reduced nuclear basophilic staining, indicating cariolysis. Cells treated with 10µg/ml of CHX for 12 hours were similar to control cells. Treatment for 24 hours induced morphologic deterioration and cariorhexis and for 48 hours led to an inhibition on fibroblast proliferation, morphologic deterioration, nuclear dislocation, cariorhexis and increased eosinophilc staining. Fluorescence was not verified on cells stained with HE, indicating that all treatments did not induce apoptosis. In conclusion, FCS starvation and CHX treatment reduce cell viability according to exposition time, causing several injuries which may reduce success of nuclear transfer.

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EFFECT OF PASSAGE NUMBER AND SEX OF FIBROBLASTS ON DEVELOPMENT OF BOVINE NUCLEAR TRANSFER EMBRYOS

Merighe, G.K.F.[1]; Miranda, M.S.[1]; De Bem, T.H.C.[1]; Watanabe, Y.F.[2]; Meirelles, F.V.[1]

[1]Laboratório de Morfofisiologia Molecular e Desenvolvimento Departamento de Ciências Básicas - FZEA/USP, Pirassununga-SP, Brasil. [2]Vitrogen - Pesquisa e Desenvolvimento em Biotecnologia da Reprodução S/C Ltda. gkrempel@usp.br

Cells cultured for long-term accumulate genetic and epigenetic alterations that result in improper nuclear reprogramming of somatic cell nuclear transfer (SCNT) embryos. Furthermore, the sex may be a limiting factor in the blastocysts production and in post-implantation developmental competence. Therefore, the objective of this study was to determine the ideal passage number to SCNT, as well, to evaluate the effect of sex on *in vitro* development and on the post-implantational competence of these embryos. Slaughtered cows derived oocytes were enucleated and reconstructed by SCNT from adult animal at 18 hours post-maturation. After fusion (2 DC pulses of 2.25 kv/cm for 65 μsec) and chemical activation (ionomycin – 5.0 μM for 5 min and 6-DMAP – 2.0 mM for 3 hours), the couplets were cultured in CR2 with monolayer of cells at 38.8ºC in a humidified atmosphere of 5% CO_2 in air for 7 days. Data were analyzed using Chi Square analysis. Blastocysts formation rate was higher on early passages (3^{rd} to 5^{th}) compared with intermediate (6^{th} to 8^{th}) and late passages (9^{th} to 12^{th}; 14%, 10% and 5%, respectively). Although the intermediate passages showed better results of pregnancy at day 30 (34%) compared with others (25% - early and 0% - late), the early passages exhibited better rates of term development (38% vs 6% - intermediate and 0% - late). Blastocysts formation showed no difference between male and female embryos (16% and 14%, respectively). Although the pregnancy rate was similar (30% and 25% respectively), the results evidenced higher competence to develop to term of male (12%, 8/66) compared to female embryos (4%, 4/107). In conclusion, these results indicates that the long-term culture of donor cells decrease the blastocyst formation and increase the chances of failure during the pregnancy, futhermore, female fetus tend to have higher rates of abortions.

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EVALUATION OF STRAWS (0,25mL) IN *IN VITRO* CULTURED BOVINE FIBROBLASTS CRYOPRESERVATION

De Bem, T.H.C.[1]; Miranda, M.S.[1,2]; Merighe, G.K.F.[1]; Watanabe, Y.F.[3]; Meirelles, F.V.[1]; Ohashi, O.M.[2]

[1] Molecular Morfofisiology and Development Laboratory - FZEA – Pirassununga-SP
[2]FIV Laboratory – UFPA – Belém-PA; [3]Vitrogen – Cravinhos-SP, tiagodebem@yahoo.com.br

The maintenance of a bank of somatic cells is very important in laboratories working on animal cloning by somatic cell nuclear transfer. In the cryo-storage, some problems like correct accommodation of cryovials inside the conventional LN_2 containers are frequently reported by many laboratories. In this study we evaluated the efficacy of the use of mini-straws (0.25mL) for freezing *in vitro* cultured somatic cells. Bovine fetal fibroblasts (FF) and adult bovine fibroblasts were loaded at the same time in cryovials (FFcryovials / FFstraws - 10^6cells/mL) and straws (FAcryovials / FAstraws - 5.10^5cells/mL) and frozen in DMEM medium supplemented with 20% FCS and 10% DMSO by gradual temperature decrease (1h 15°C, 1h in freezer -20°C, 48h in freezer -86°C) followed by LN_2 immersion. The cells were thawed right away at room temperature and a part was evaluated for cell membrane integrity using Hoescht 33342 (5µg/mL) and propidum iodide (100µg/mL) dyes. The remainder cells were plated on culture dishes and analyzed for fixation and multiplication. Chi-square was used for statistical analysis. The FFstraw group (n=228) presented a higher number of dead cells compared with the FFcryovial group (n=514; 19.7% vs. 13%, respectively). Adult somatic cells showed the same pattern observed in fetal cells, with an increased number of cell death in FAstraws (n=306) compared with FACryovials (n=405). Although straws resulted in a 10% decrease in cellular viability, bovine fetal and adult fibroblasts loaded in straws allowed cell attachment and replication after thawing. There is, however, a need to determine in further experiments if the higher frequency of cryoinjury leads to other cells alterations, such as those related to chromosomal defects.

PRELIMINARY RESULTS OF BUFFALO CYTOPLASMIC TRANSFER TO BOVINE ZYGOTES

Ferreira, C.R.[1]; Meirelles, F.V.[2]; Sá Filho, M.F.[3]; Baruselli, P.S.[3]; Gasparrini, B.[5]; Perecin, F.[1]; Méo, S.C.[4]; Tetzner, T.A.D.[1]; Saraiva, N.Z.[1]; Yamazaki, W.[1]; Vantini, R.[1]; Garcia, J. M.[1]

[1]DMVPRA FCAV-UNESP, 14884-900, Jaboticabal-SP, Brazil, [2]FZEA-USP, ZAB, Pirassununga-SP, Brazil, [3]DRA-USP, São Paulo-SP, Brazil, [4]CPDGRA-IZ, APTA/SAA, Nova Odessa-SP, 13460-000, Brazil. [5]University of Frederico II, Napoly, Italy. chrisferreira13@yahoo.com.br

Interspecific zygote cytoplasmic transfer (CT) can be used to study nuclear-cytoplasmic compatibility, epigenetic effects on embryo development and phenotypical effects of mtDNA. It may also be employed to evaluate the possibility of rescuing endangered species by using receptor cytoplasm from closely related domestic species for nuclear transfer. Our aim was to apply and to evaluate interspecific CT of buffalo to bovine zygotes. Bovine oocytes obtained from slaughtered cows were *in vitro* matured (IVM) in groups of 25 in 100μL drops of bicarbonate-buffered TCM-199 supplemented with 10% FCS and hormones for 24h under mineral oil at 38.5°C in a atmosphere of 5% CO_2 in air and fertilized to produce receptor zygotes. Buffalo oocytes to be used as cytoplast donors were collected through Ovum Pick-up (OPU), *in vitro* matured in the same conditions used for bovines, but IVM medium was supplemented with 50μM of cysteamine and 0.3mM of cistine. After 22h IVM, buffalo oocytes were enucleated in MII and parthenogenetically activated (ionomycin 5 μM for 5 min and 6-DMAP 2 mM for 4 hours). After 10-12h IVF bovine receptor zygotes were washed 3 times in HEPES-buffered SOF supplemented with 10% FCS, had their *cumulus* cells removed with hialuronidase 0.5% and were selected for the presence of the second polar body (2nd PB). Microsurgery system consisted of two holding pipettes (one for the cytoplasm donor and the other for the receptor zygote) and one injection pipette. Both bovine receptor zygotes and buffalo donor cytoplasts were placed during 30 min in medium containing 7.5μg/mL cytochalasin B (CB) before microsurgery, that was performed in HEPES-buffered SOF supplemented with 10% FCS and 7.5μg/mL CB. Bovine zygotes had their 2nd PB and surrounding cytoplasm removed previously to CT. Around 10-15% of buffalo cytoplasm was introduced into the periviteline space of the receptor bovine zygote and electrofused (2 pulses of 1.5kV/cm for 30μs in 0.28M mannitol solution). Sixteen (61.53%) out of 26 interspecific CT embryos cleaved and 3 blastocysts developed (11.54%). The 3 blastocysts were transferred at day 7 to synchronized surrogate cows, resulting in one pregnancy (going on) that is closely to term. IVF control (n=30) resulted in 70% cleavage and 10% blastocyst development. These preliminary results suggest that transferring 10-15% buffalo cytoplasm to bovine zygotes do not disturb embryo development, but further studies are need to confirm this hypothesis. Buffalo interspecific CT to bovine zygotes is compatible with gestation. Post-fusion embryos and the animal/fetus delivered will be analyzed to estimate the amount of Buffalo mtDNA introduced and to study it fate during fetal development.

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PARTHENOGENETIC ACTIVATION OF BOVINE OOCYTES WITH CALCIUM IONOPHORE A23187 (CA) OR IONOMYCIN (IO)

Milazzotto, M.P.; Feitosa, W.B.; Simões, R.; Martins, L.F.; Coutinho, A.R.S.; Assumpção, M.E.O.A.; Visintin, J.A.

Departament of Animal Reproduction – FMVZ-USP, Sao Paulo-SP, Brazil
mazamila@terra.com.br

Activation of *in vitro* maturated oocytes is essential for the success of cloning by nuclear transfer. For using calcium ionophore A23187 (CA) and ionomycin (IO). The combination of CA with 6-DMAP induces high rates of activation and development to blastocyst. However, oocytes activated with IO and 6-DMAP may lead to some alterations in the DNA content, reflecting an abnormal pattern of karyokinesis during the first cell cycle. The aim of this work was to evaluate the effect of calcium ionophore A23187 or ionomycin as primary agents of bovine oocyte activation, associated or not to BSA, an activation blocker, on embryo rates and quality. Cumulus-oocytes complexes were maturated in TCM199 medium with FCS and hormones for 18hs at 38.5ºC and 5%CO_2 in air. After denuded, oocytes presenting the first polar body were selected and maintained in SOFaa medium to complete 24hs from the beginning of maturation and were divided in four groups: 1-CA (CA 5mM, 5min + 6-dimethylaminopurine (6-DMAP), 3hs); 2 - CA (CA 5mM, 5min + BSA, 5min + 6-DMAP, 3hs); 3 - IO (IO 5mM, 5min + 6-DMAP, 3hs); 4 - IO (IO 5mM, 5min + BSA, 5min, + 6-DMAP, 3hs). After treatments, oocytes were cultured in SOFaa medium for 7 days at 38.5ºC and 5%CO_2 in air. Cleavage and blastocyst rates were evaluated respectively, on days 2 and 7 of culture. To evaluate embryo quality, Hoechst 33342/ propidium iodide staining was used. Datas were evaluated by ANOVA and submitted to LSD test for embryo rates and T test for embryo quality. Four replicates were done with a total of 89 oocytes per treatment. There was difference ($p<0.05$) in embryo rates between groups 1 - CA (54.4%^{a}) and 3 - IO (51.4%^{a}) comparing with group 4 - IO (18.3%^{b}). Group 2 - CA (39.8%^{ab}) did not show any difference from the others. However, there was no difference ($p>0.05$) in number and percentage of viable cells between groups 1 - CA (63.1 and 49.9%), 2 - CA (57.2 and 45.8%), 3 - IO (60.9 and 64.9%) and 4 - IO (72.4 and 50.9%), respectively. We can conclude that adittion of BSA was not beneficial for oocyte acivation and had a negative effect when associated with IO on embryo rates. However, the different calcium ionophore used and the BSA did not improve embryo quality.

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ACTIVATION AND PARTHENOGENETIC DEVELOPMENT OF BOVINE YOUNG OOCYTES

Biondi, F.C.[1]; Cordeiro, M.S.[1]; Miranda, M.S.[1]; Santos, S.S.D.[2]; Ohashi, O.M.[1]

[1] Laboratório de Fertilização *in vitro* - CCB/UFPA, Belém-PA, Brasil [2] Departamento de Histologia e Embriologia - CCB/UFPA, Belém-PA, Brasil fbiondi@ufpa.br

In the field of the reproductive biotechnology, the development of effective parthenogenetic activation protocols is useful for the study of the oocyte competence and essential to improve nuclear transfer procedures in domestic species. In mammalian, the parthenogenetic activation can be induced by several agents as follow: electric pulse, ethanol, calcium ionophore, strontium, ionomycin, and a combination of these agents with protein synthesis or protein phosphorylation inhibitors as cycloheximide and 6-dimethylaminopurine (6-DMAP). The present study aimed to evaluate the chemical activation with ionomycin and strontium, and the effect of 6-DMAP on the activation and embryonic development of young bovine oocytes. Bovine oocytes from local slaughterhouse were maturated *in vitro* in TCM199 with bicarbonate, FSH, LH, insulin, cysteamin, pyruvate and antibiotics, in 5%CO_2, 38.5ºC and humidified air, during 24 hours. After IVM, the oocytes were denuded and only those having the first polar body were exposed to ionomycin 5 μM (I) for 5 minutes or strontium 25 mM (S) for 6 hours. After that, they were incubated in CR2 medium (I and S) or CR2 + 2.0 mM of 6-DMAP (ID and SD). After 4 (ID and SD) and 15 hours (I and S) of culture, the oocytes were fixed and the pronuclear formation was evaluated. Cleavage and embryonic development to blastocyst stage were analyzed on the 2nd and 8th day of culture, respectively. Comparisons between groups were performed by chi-square test, employing the software CLUMP. Differences were considered to be significant at $p < 0.05$. The results showed successful activation of young bovine oocytes by combining treatments of ionomycin or strontium and 6-DMAP. The incubation with 6-DMAP increased ($p<0.05$) the pronuclear formation, cleavage and blastocysts rates of ionomycin (18.1% *vs.* 99.1%; 5.8% *vs.* 65.2%; 0% *vs.* 44.3%) and strontium (33.8% *vs.* 81.7%; 35.3% *vs.* 54.3%; 4.3% *vs.* 25%). Strontium pronuclear formation and cleavage rates were higher ($p<0.05$) than the ionomycin treatments, in absence of 6-DMAP (33.8% *vs.* 18.1% and 35.3% *vs.* 5.8%); but, in combination with 6-DMAP, ionomycin pronuclear formation and blastocysts rates were higher ($p<0.05$) than strontium groups (99.1% *vs.* 81.7% and 44.3% *vs.* 25%). The highest ($p<0.05$) pronuclear formation, cleavage and blastocysts rates were obtained in ionomycin plus 6-DMAP treatment, with those similar to IVF. Additionally, the results show that treatments with ionomycin or strontium induce low rates of pronuclear formation, cleavage and blastocysts, and for better results for young bovine oocytes activation is necessary the treatment with protein synthesis or phosphorylation inhibitors to prevent the reactivation of MPF.

PARTHENOGENETIC DEVELOPMENT OF BOVINE OOCYTES BLOCKED WITH BUTYROLACTONE I: PRELIMINARY RESULTS

Miranda, M.S.; Merighe, G.K.F.[1]; Adona, P.R.[1,2]; Biase, F.H.[1]; Meirelles, F.V.[1]; Leal, C.L.V.[1]

[1]Departamento de Ciências Básicas – FZEA/USP – Pirassununga-SP, [2] Setor de Reprodução Animal – LMGA/CCTA/UENF – Campos dos Goytacazes-RJ

In order to improve cattle *in vitro* production results, a pre-maturation culture using cyclin dependent kinase inhibitors has been proposed. The meiosis block would allow additional time for the oocyte to improve cytoplasmic maturation and increase the competence for embryo development. The aim of this work was to assess the competence for parthenogenetic embryo development in bovine oocytes blocked with butyrolactone I (BL). Oocytes, collected by follicular aspiration (2-6mm) from cow ovaries after slaughter were split into two groups: G1 – blocked with 10µM BL in TCM199 with 10µg/mL gentamycin for 24h and then *in vitro* matured for 18h (TCM199 with 10% FCS, 5.0µg/mL LH, 0.5µg/mL FSH and 10µg/mL gentamycin); G2 – oocytes matured *in vitro* for 18h without prior use of inhibitor (control). After maturation, oocytes were denuded and selected for the presence of the first polar body (PB) and activated with 5µM ionomycin in TCM199 for 5 min followed by incubation in 2mM 6-DMAP diluted in culture medium (SOF with 10% FCS) for 3h. Activated oocytes were cultured in SOF for 7 days on a monolayer of granulosa cells in 5% CO_2 in air. Preliminary results show that there was no difference ($P>0.05$) between G1 (n = 51) and G2 (n = 44) regarding PB extrusion rates (67% vs. 75.1%, respectively), cleavage rates (61% vs. 59%), 8-cell embryos at 48h post-activation (35.4% vs. 38.4%) and embryo development to the blastocyst stage 192h after activation (21.5% vs. 34%). Therefore, meiosis blockage using BL for 24h apparently does not increase developmental competence of bovine oocytes. As a negative effect was also not observed, the use of this protocol could be useful for manipulating maturation time in order to maximize the use of oocytes in somatic nuclear transfer programs.

FFICIENCY OF CHEMICAL OR ELETRICAL ACTIVATION OF BOVINE OOCYTES

Milazzotto, M.P.; Feitosa, W.B.; Martins, L.F.; Yamada, C.; Nicacio, A.C.; Assumpção, M.E.O.A.; Visintin, J.A.

Departamento de Reprodução Animal – FMVZ-USP, São Paulo-SP, Brasil
mazamila@terra.com.br

Chemical oocyte activation can be induced by ionophore A23187 (CA) that promotes the release of intracellular calcium and also facilitates the influx of extracellular calcium ions, important for nuclear transfer. Eletrical stimulation (EL) is an essential stage for nuclear transfer protocols. To fusion of the enucleated oocyte cytoplasm with donor nucleus cell. Moreover, EL can be used as an alternative method than chemical activation to induce calcium influx through the formation of pores in the plasma membrane. The aim of this work was to verify three different protocols using eletrical activation (EL), chemical activation (CA) or both (CE) on embryo rate and qualityu. Cumulus-oocytes complexes were maturated in TCM199 medium supplemented with FCS and hormones for 18hs at 38.5ºC and 5%CO_2 in air. After denuded, oocytes presenting the first polar body were selected and maintained in SOFaa medium to complete 24hs from the beginning of maturation and than divided in three groups: 1-CA (CA 5mM, 5min + 6-dimethylaminopurine (6-DMAP), 3hs); 2- EL (EL 1.5kV/cm, 20µs, 2 pulses + 6-DMAP, 3hs); 3-CE (EL 1.5kV/cm, 20µs, 2 pulses + CA 5mM, 5min + 6-DMAP, 3hs). After treatments, oocytes were cultured in SOFaa medium for 7 days at 38.5ºC and 5%CO_2 in air. Cleavage and blastocyst rates were evaluated respectively, on days 2 and 7 of culture. For embryo quality, Hoechst 33342/ propidium iodide staining was used. Datas were evaluated by ANOVA and submitted to LSD test for embryo rates and T test for embryo quality with 5% of significancy. Four replicates were done with a total of 89 oocytes for group 1, 79 for group 2 and 120 for group 3. There were no differences ($p>0.05$) on embryo rates among groups 1 - CA (54.3%), 2 - EL (54.5%) e 3 - CE (32.8%). There were no differences ($p>0.05$) in the embryo cell number between groups 1 - CA (64.6), 2 - EL (58.4) e 3 - CE (52.7). However, there was difference ($p<0.01$) in percentage of viable cells between groups 1 - CA (50%^{a}) and 2 - EL (82.7%^{b}), but not in group 3 - CE (63%^{ab}). We can conclude that, although electrical stimulation induce calcium influx and oocyte activation did not increase embryo rates, but it can be an alternative to produce NT embryos with higher quality.

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HATCHED RATES OF MICE EMBRYOS OF C57BL/6 AND C57BL/6 TRANSGENIC STRAINS AFTER *INVITRO* CULTURE

Maffili, V.V.[1]; Paiva, F.P.[1]; Silva, C.A.[1]; Viana, J.H.M.[2]

[1] Centro de Pesquisa Gonçalo Moniz – FIOCRUZ – vmaffili@cpqgm.fiocruz.br; [2]Centro de Ensino Superior de Juiz de Fora

The objective of this study was to compare the *In vitro* development of *wild type* (*wt*) C57Bl/6 mice embryos and certain transgenics strains in *background* C57Bl/6, after culture. In the present study were used embryos from six weeks' old females C57Bl/6 *wt* (T1) and others with the following transgenic strains: C57Bl/6 iNOStm1Plh (T2), C57Bl/6 Ifngr^{1tm1Agt} (T3), C57Bl/6 GFP (T4), C57Bl/6 Gt(ROSA)26Sor^{tm1Ts} (T5), C57Bl/6 Tnfrsf1a^{tm1Imx} (T6), C57Bl/6 Cd28^{tm1Mak} (T7), C57Bl/l Il10^{tm1Cgn} (T8) and C57Bl/6 ß2mtm1Unc (T9). The females were super-ovulated with the administration of 5UI of eCG, followed by 5UI of hCG, with 48 hours' intervals. Soon after the application of hCG, the females were bred at 1:1 ratio and removed in the following day. Embryos were collected 38 hours after the application of hCG. The embryos were obtained by flushing the uterine tube with M2 medium, selected and classified. Viable embryos were placed in a crioprotector solution (1,5M propileneglicol in M2 medium) for 15 minutes. After this period, the embryos were frozen in a programmable freezer at -7^{o}C. After 5 minutes, "seeding" was performed and the embryos submitted to a cooling curve of 0,3^{o}C / min until -30^{o}C, being then immersed in N_2 and stored until thawed (RENARD, J.P.; BALBINET, C.; J. Exp. Zoo., 230-443, 1984). Thawing was realized by removing the straw, leaving it exposed to room temperature for 40 seconds. Then, the content of the straw was expelled in a Petri dish of 35mm and left for 5 minutes. After this period, the embryos were transferred to a plate in M2 medium and then cultured in M16 medium for 72 hours in atmosphere contend 5% of CO_2, 5% of O_2 and 90% of N_2 in a temperature of 37^{o}C. Development rate until blastocist stage was verified carried out through a confrontation of the data in the contingency table and using the Chi-square test with 5% probability. Hatched rates were: T1 = 46/66 (69.7%)^{a}; T2 = 20/27 (54.4%)^{a,b}; T3 = 20/47 (42.5%)^{b,c}; T4 = 30/55 (54.6%)^{a,c,d,e}; T5 = 25/44 (56.8%)^{a,c}; T6 = 33/51 (64.7%)^{a}; T7 = 18/38 (47.4%)^{b,d}; T8 = 21/59 (35.6%)^{b,e,f} and T9 = 8/42 (19.0%)^{f}. It can be concluded that, embryos of transgenic mice C57B1/6 strains present reduced development rates *in vitro*. However, the factors involved in this reduction still need further research.

BOVINE SPERM CAPACITATION USING CALCIUM IONOPHORE FOR *IN VITRO* PRODUCTION OF TRANSGENIC EMBRYO

Simões, R.; Binelli, M.; Mendes, C.M.; Coutinho, A.R.S.; Feitosa, W.B.; Braga, F.A.; Visintin, J.A.; Assumpção, M.E.O.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
resimoes@usp.br

In vitro production (IVP) of transgenic embryos is acomplished in mice by pronuclei microinjection, but in bovine this is not a successful technique because oocytes have a great amount of lipids. Sperm-mediated gene transfer (SMGT) is an alternative to this problem, has lower cost and does not require microinjection handling. One way to introduce exogen DNA into oocytes is via sperm capacitated with calcium ionophore (CaI). The aim of this work was to evaluate different CaI concentrations ([CaI]), time of sperm incubation with CaI (tCa) and incubation time of capacitated sperm with DNA (tDNA) (EYFP, Clonetech, USA) to establish a satisfactory method for IVP of transgenic embryos. Slaughterhouse oocytes with compact *cumulus* and uniform ooplasm were *in vitro* maturated in TCM-199 medium + 10% FCS + FSH + HCG + E^2 + piruvate + gentamicine under 5% CO_2 in air, at 39ºC and high humidify for 24hs. Semen was thawed in water at 37ºC/30 seconds and separated in a Percoll gradient (45/90%) at 600g/30 min. After this procedure, sperm cells were washed in Talp-semen medium by centrifugation at 200g/5min at 25ºC. Supernatant was removed and capacitation was induced with CaI (250nM or 500nM for 1or 5 min) in $5x10^6$ spermatozoa/group. Capacitated sperm cells were incubated with DNA for 1 or 2hs. Non-treated spermatozoa were used as control group. Sperm cells ($1x10^5$) were used to inseminate 20 oocytes/microdroplet for 18hs. The presumptive zygotes were co-cultured in SOFaa medium with a granulosa cell monolayer under high humidified atmosphere, at 39ºC and 5% CO_2 in air. Blastocyst rates (%BL) were analysed by ANOVA. Independent variables were replicates, [CaI], tCa, tDNA and double and triple interactions among the last three variables and, when apropriate, means were compared by orthogonal contrasts. There was [Ca] x tCa x tDNA interaction for %BL ($p<0.02$). Treatments with 250nM [CaI], 5min tCa, 1h tDNA or 500nM [CaI], 1min tCa, 1h tDNA showed %BL of 36.1% and 37.4%, respectively, similar to control group (30.5%, $p>0.4$). In conclusion, these results demonstrated that it is possible to capacitate spermatozoa with CaI to produce transgenic embryos, without alteration of blastocyst rate.

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PRONUCLEAR TRANSFER TO ACESS NUCLEAR-CYTOSPLASMIC INTERACTION BETWEEN BOVINE OOCYTES FERTILIZED AND ACTIVATED WITH STRONTIUM

Méo, S.C.[1,2]; Ferreira, C.R.[2]; Perecin, F.[2]; Yamazaki, W.[2]; Leal, C.L.V.[3]; Meirelles, F.V.[3]; Garcia, J.M.[2]

[1]Centro de Genética e Reprodução - Instituto de Zootecnia, APTA/SAA, Nova Odessa-SP, 13460-000, Brazil; [2]Departamento de Medicina Veterinária Preventiva e Reprodução Animal - FCAV/UNESP, Jaboticabal-SP, 14884-900, Brazil; [3]Departamento de Ciências Básicas – FZEA/USP, Pirassununga-SP, 13635-900, Brazil. simone@iz.sp.gov.br

For domestic animals, the search to improve production rates and to disseminate animals of high genetic merit gave rise to the development of several reproductive biotechnologies, among them, embryo *in vitro* production and cloning. Somatic cell nuclear transfer is a very important tool and its success depends on receptor cytoplasm ability in inducing somatic cell reprogramming to create a new individual. In this paper, we aimed to better understand the nuclear reprogramming events by evaluation of the nuclear-cytoplasmic interaction effect on preimplantation embryonic development. Bovine oocytes were *in vitro* matured for 24 h in TCM199 supplemented with 10% FCS, pyruvate and hormones, and then they were fertilized (IVF) or artificially activated with ionomycin (5 µM for 5 min) and strontium (20 mM for 6 h; IS). After 24-30 h, presumptive zygotes were centrifuged at 15.000 xg for 15 min for pronuclear visualization and designated to pronuclear transfer (PT) procedures. The results were analyzed by Chi-square test (p=0.05). IS and micromanipulated control IS embryos (PT-IS; produced after removal and transfer of the same pronucleus to the same activated zygote) showed similar cleavage (77.9 and 73.6%) and blastocyst development (7.8 and 7.3%) rates. However, cleavage and development rates for IVF (69.6 and 21.7%) were superior to micromanipulated control IVF (PT-IVF; produced after removal and transfer of the same pronucleus to the same IVF zygote; 56.2 and 2.2%). This demonstrates the negative impact of the PT technique in IVF embryos when compared to IS embryos. Cleavage and blastocyst development rates for embryos reconstructed with IVF cytoplasm and IS nucleus (CFNS; 59.8 and 1.4%) and with IS cytoplasm and IVF nucleus (CSNF; 51.2 and 1.6%) were similar to PT-IVF, but they were inferior to PT-IS, confirming the difficulty to perform PT in IVF embryos. When CFNS and CSNF embryos were compared, similar cleavage and blastocyst development rates were observed. These results indicate that the IVF cytoplasm was able to support the development of the IS nucleus as much as the IS cytoplasm was able to support the IVF nucleus. However, besides the influence of the pronuclear transfer procedures on both embryos, the results suggest that nuclear-cytoplasmic incompatibility between IVF and IS embryos may exist, at least during early embryonic development, since cleavage rates for CFNS embryos tended to be higher (p=0.051) than the rates observed for CSNF embryos. Thus, we conclude that pronuclear transfer can be a useful tool for evaluation of nuclear-cytoplasmic interaction, but if it is improved enough to avoid the influence of the technique on the development of IVF reconstructed embryos.

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SENSIBILITY EVALUATION OF MOUSE ZYGOTES TO *Brucella abortus* FOR ESTABLISHMENT OF AN EXPERIMENTAL MODEL TO PATHOGEN-EMBRYO INTERACTION STUDIES

Galuppo, A.G.; D'Angelo, M.; Miyashiro, S.; Scarcelli, E.; Senedezi, M.L.P.; Melo, G.M.

Centro de Sanidade Animal-Instituto Biológico de São Paulo, São Paulo-SP, 04014-002, Brasil
andrea_giannotti@hotmail.com

In the epidemiologist's point of view, broad implementation of new animal reproductive techniques represents a challenge by contributing to animal pathogens dissemination. Pathogens infecting the ovary, uterus or oviduct tissues are the most relevant in those cases, which led to the concerne of the *Brucella abortus* transmission via animal reproductive techniques. The aim of this study was evaluate *in vitro* mouse zygotes sensitivity to *Brucella abortus*, and the efficacy of embryo washing procedure and trypsin treatment, for its removal and/or inactivation, with the purpose of establishing an accessible model for embryo-pathogen interaction studies. Female mice (Swiss Webster) aging 6 to 8 weeks were superovulated and matted with fertile males of the same strain, then the zygote retrieval was performed. The bacterial suspension was prepared in the moment of inoculation in a dilution of 10^6 brucellas/mL. The zygotes were divided into control and infected groups (30µl of the bacterial suspension); after 24h and 96h their morphology and viability were analised. They were kept in HTF medium (CULTILAB®-Campinas, SP), supplemented with 10% fetal calf serum (CULTILAB®-Campinas, SP). The zygotes of each group were washed sequentially or treated with trypsin after 24h exposition. To verify the presence of *B. abortus* pos-washing the zygotes and a sample of the last wash drop of each group were tested on bacterial culture system and polimerase chain reaction (PCR). The statistical analyses was performed with the χ^2 test. Morphological changes were not observed at the control group, but the infected one presented irregular blastomeres, clivage defective, granular cytoplasm with degenerative like morphology. The clivage rates were 77.4% (control) and 59.2% (infected) (χ^2 of 0.001674; $p<0.05$) after 24h and after 96h 14.5% (control) and 7% (infected) (χ^2 of 0.141616; $p<0.05$). Bacterial culture presented negative growing for all groups tested. The control group presented only negative results on PCR analyses. The infected group presented positive and negative results on PCR, for embryos or last wash drop sample, submitted to the sequential washing procedure, respectively. Positive embryos were found in just one sample with the last wash drop negative. To two other samples embryos presented negative PCR results but positive at the last wash drop. Negative results for embryos and last wash drop was found in just one sample. The PCR results for the groups treated with trypsin were almost all positives, for embryos or last wash drop samples, except one embryo sample that was negative. According to our results murines embryos could be consider sensible to *Brucella abortus*. The recent developed reproductive technologies promoted excessive manipulation of the embryo. The preeminent risk to be considered here is the potential or probability of the presence of pathogens associated with or in proximity to the zona pelúcida, which could be introduced, or facilitated its entrance on embryo. According to the data presented become clear the importance of develop a model for studies of embryo-pathogen interactions, with the aim of avoid disease transmission.

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CHARACTERIZATION OF THE TOP OF THE MATERNAL VILLI AND THE BASIS OF THE FETAL VILLI IN ZEBÚ CATTLE (*Bos indicus* - LINNAEUS, 1758)

Pereira, F.T.V.[1]; Cazerta, S.M.M.[1]; Miglino, M.A.[2]; Braga, F.C.[2,3]; Meirelles, F.V.[3]; Assis Neto, A.C.[1]; Marques, R.S.[1]; Vulcano, M.

[1]Faculty of Zootecny of UNESP - *Campus* Dracena, Dracena-SP, 17900-000, Brazil. [2]Faculty de Veterinary Medicine and Zootecny of USP (FMVZ-USP/SP), Sao Paulo-SP, Brazil. [3]Faculty of Zootecny and Food Engineering of USP (FZEA-USP), Pirassununga-SP, Brazil.
fverechia@dracena.unesp.br

The specific region of the top of the maternal villi and the basis of the fetal villi in bovines was not very studied, mainly in Zebu cattle and corresponds to the arcade zone of the sheep placentome, were there are areas with blood extravasations (haemophagous areas) and areas without this process, which is realized by the trophoblast. It is possible that the haematome is resulted of the opening of the maternal capillaries in the top of adjacent trophoblast intervillous septa and presents the function to supply the source of the iron to the fetus. Some studies have demonstrated that the deficiency of iron during the gestation results in the long term problems in the neonate, as for example increase in the blood pressure, reduction of the cerebral function and compromised of the development of the immune system. Our objective was to characterize the haemophagous areas of the bovine materno-fetal interface. For this work we utilized placentomes of 30 zebu cows collected in slaughter-houses in the period of 4-8 months of gestation, fixed by immersion with 10% formaldehyde and 4% paraformoldehyde in PBS pH 7,4 0,1 M, processed and stained for light microscopy (H&E, picrosirius, Masson's trichrome) to observe the morphology and histochemistry reactions (PAS, Perls) to observe the cells activities related to absorption, transport of molecules and presence of secretions. The materno-fetal interface in bovines is constituted by the trophoblast (fetal part) and by the uterine epithelium (maternal part). The trophoblast is constituted by a single layer and presents two distinct cells populations, the epithelial cells and the uni, bi or multinucleate cells, being the binucleate the most common. The maternal epithelium is constituted by cuboid type epithelium. In the amounts collected from the six months of gestation we detected the occasional accumulate of the maternal blood extravasation in the materno-fetal interface with consequent trophoblastic erythrophagocytosis. The haematome aspect remained the same in all phases of pregnancy showing a great morphologic variation. When present these haematomes were located mainly in the apex of the maternal villi, what it corresponded to the basis of the chorionic villi. The trophoblastic cells presented erythrocytes in its interior, suggesting a possible transference of iron for the embryo. The binucleate trophoblastic cells had not been visualized realizing phagocytosis. We believe that the embryo uses this extravasated ion of the mother for its hematopoiesis. In the extravasated blood of the haematome cellular debris and probable cells in apoptosis had also been observed. In the region of the haematome, vesicles and granules in the trophoblastic cells had been observed. The results obtained by the light microscopy had indicated that these haemophagous areas of the maternal-fetal interface of bovines are very important sites of iron transfer and can be involved in regulation of fetal hematopoiesis. These data can be transferred to bovines placenta manipulated in laboratory as form of relief in the detection of many gestation losses and placental alterations.

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CHARACTERIZATION OF THE INTERCARUNCULAR REGION OF THE PREGNANT ZEBÚ COWS (*Bos indicus* – Linnaeus, 1758).

Marques, R.S.[1]; Miglino, M.A.[2]; Braga, F.C.[2,3]; Meirelles, F.V.[3]; Assis Neto, A.C.[1]; Vulcano, M.[1]; Cazerta, S.M.M.[1]; Pereira, F.T.V.[1]

[1]Faculty of Zootecny of UNESP - *Campus* Dracena-, Dracena-SP, 17900-000, Brazil. [2]Faculty de Veterinary Medicine and Zootecny of USP (FMVZ-USP/SP), Sao Paulo-SP, Brazil. [3]Faculty of Zootecny and Food Engineering of USP (FZEA-USP), Pirassununga-SP, Brasil.
rodrigomarques@dracena.unesp.br

The ruminant placentation is referred to the junction of the chorion with the uterine epithelium resulting in the functional placenta and in the placentomes, which are the morphofunctional unities of it. In the ruminant uterus surface there are non-glandular projections denominated caruncles that together with the chorionic villosities give origin to the placentomes. The regions among these caruncles are called intercaruncular regions and are composed by the perimetrium, miometrium and endometrium, which presents a glandular structure that, represents an important role in the estrus cycle and in the preparation of the uterus to the implantation of the blastocyst. Leading in consideration that these glands play the role of the iron transport and another substances of the mother to the fetus, the objective of this work is to supply informations about the development of the intercaruncular regions of the bovine placenta throughout all the gestation, and to apply them in biotechnology researches on animal reproduction. For this work were utilized intercaruncular regions of the pregnant uterus of 55 zebu cows collected in slaughter-houses in the period of 4-8 months of gestation, fixed by immersion with 10% formaldehyde and 4% paraformoldehyde in PBS pH 7.4 0.1 M, processed and stained for light microscopy (H&E, picrosirius, Masson's trichrome) to observe the morphology and histochemistry reactions (PAS, Perls). The obtained results showed that the intercaruncular regions were situated among the uterine caruncles in the non-pregnant and in pregnant uterus, which had been presented very developed in this last one. The caruncles are materno-fetal interchange areas and presents in all the surface crypts with honeycomb aspect, however, with the gestation progress presents big caruncles in the pregnant uterine horn than in the non-pregnant uterine horn, can occur fusions with adjacent caruncles to become greaters and together with the chorionic villi connection, leading to the placentome formation. The intercaruncular regions are composed by the endometrium, constituted by the simple cuboid epithelium to simple columnar type, the basal lamina (sub mucous layer) composed by dense connective tissue, the miometrium composed by internal and external circular layer of smooth muscle and the perimetrium, serous layer with presence the many blood vessels. The endometrial glands are constituted by simple columnar epithelium and are found in the endometrial stroma in high quantity, but can advance until the miometrium and with the gestation progress they visually increase your size and number and are important sites of the substance transfer from mother to the fetus. In the gland lumina had cellular debris and mucoid secretions PAS positives. The endometrial stroma presented a considerable number of fibroblasts and blood vessels and below the endometrium we visualized the miometrium composed by smooth muscle fibers. According to the utilized methodology we could conclude that the endometrial glands are important sites of the secretion and transfer of substances from the mother implicated in the nutrition of the bovine fetuses.

Sponsors: CNPq PIBIC

IMMUNOHISTOCHEMICAL STUDY OF THE TOP OF THE MATERNAL VILLI AND BASIS OF THE FETAL VILLI REGION OF THE WATER BUFFALO PLACENTA (*Bubalus bubalis bubalis* - Simpson, 1945)

Burioli, K.C.[1]; Miglino, M.A.[2]; Braga, F.C.[2]; Bazer; F.W.[3]; Kfoury Jr., J.R.[2]; Oliveira, L.J.[2]; Papa, P.C.[2]; Oloris, S.C.S.[2]; Dagli, M.L.Z.[2]; Carvalho, A.F.[4]; Pereira, F.T.V.[1]

[1]Faculty of Zootecny of UNESP - *Campus* Dracena-, Dracena-SP, 17900-000, Brazil. [2]Faculty de Veterinary Medicine and Zootecny of USP (FMVZ-USP/SP), Sao Paulo-SP, Brazil. [3]Texas A&M University – USA. [4]UNIFEOB (Sao Joao da Boa Vista/SP)
kburioli@yahoo.com.br

The transplacental transport of iron by the uteroferrin (Uf), and the haemophagous areas in the water buffalo placenta was analyzed due to clarify this mechanism of blood extravasation in the materno-fetal interface (haemophagous areas) with consequent transfer of iron to the fetus through the trophoblastic erytrophagocytosis (Murai; Yamauchi, 1986). In the water buffalo placenta this mechanism remains unclear and the uteroferrin is very important in this process because is an iron transporter and a progesterone-induced hematopoietic growth factor. Our objective was to characterize these haemophagous areas in the water buffalo placenta, focusing in the materno-fetal transfer of iron, because providing adequate nutrition to the fetus is a key to a successful pregnancy. Small pieces of the water buffaloes placentomes (*Bubalus bubalis bubalis*, *n*=51) in all of the gestation periods were fixed in 10% formaldehyde and 4% paraformoldehyde in PBS, processed and stained for light microscopy to characterize the haemophagous areas morphologically and immunohistochemistry with the rabbit anti pig uteroferrin antibody to confirm the iron transfer. The haemophagous areas were present in the placentome from 4-10 months pregnant placentae and were characterize in a light microscope how areas of punctual blood extravasation between the uterine and trophoblastic epithelium by an erosion of the maternal capillaries. The immunohistochemical reaction with the anti-uteroferrin antibody was positive in the trophoblast, mainly in determined regions of the materno-fetal interface were the extravasated blood was in direct contact with the trophoblastic epithelium and in other points deep in the placentome. The results obtained by the imunohistochemistry with the specific antibody proved that the placentome haemophagous areas are very important sites for transfer of transplacental substances evolved in histiotrophic nutrition of water buffalo fetus.

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EFFECT OF COX-1 AND 2- SELECTIVE INHIBITORS COTREATMENT ON FETAL IMPLANTATION.

Tomé, A.R.[1]; Lima-Júnior, R.C.P.[2]; Cunha, K.M.A.[2]; Santos, F.A.[2]; Rao, V.S.N.[2]

[1] Faculdade de Veterinária –UECe, Fortaleza-CE, Brasil. [2]Departamento de Fisiologia e Farmacologia – UFC, Fortaleza-CE, Brasil. adrtome@yahoo.com.br

Cyclooxigenase, COX-1, a constitutive enzyme, is thought to serve "housekeeping" functions. COX-2, however, is highly inducible by diverse stimuli including cytokines, growth factors. Moreover, it regulates, for instance, inflammation and angiogenesis processes. Ovulation and implantation are considered analogous to "proinflammatory" responses. Thus, this work aimed to evaluate the effect of COX-1 and 2 selective inhibitors on blastocyst implantation phase in pregnant rat. Female rats (n=6), Wistar, were placed in polyethylene cages with male rats (2:1) and 14 h later, the vaginal fluid was collected to confirmation of pregnancy (visualized for the presence of spermatozoids, and the first day of pregnancy was then determined). At the 4, 5 and 6 days, the animals were orally treated with vehicle (distilled water), indomethacin (IND 5 mg/kg – PRODOME, Campinas-SP, Brazil), rofecoxib (ROF 5 mg/kg – Merck, São Paulo-SP, Brazil), IND (2.5 mg/kg) + ROF (2.5 mg/kg) ou IND (5 mg/kg) + ROF (5 mg/kg), with 30 min of interval in case of cotreatment. At day 10, the animals were sacrificed by cervical dislocation, the uterus horns exposed and then the total number of implantation sites and corpus luteum were counted. Statistical sinificance ($p<0.05$) was analysed by ANOVA and Student-Newman Keuls as a post-test. Results were expressed as mean ± SEM. The results indicated that the animal treatment with IND 5 mg/kg + ROF 5 mg/kg induced a significant reduction ($p<0.001$) on the number of implantation sites (0.0 ± 0.0) when compared to the vehicle-treated group (9.0 ± 1.34). Moreover, there was a significant icrease ($p<0.05$)) on the number of corpus luteum at the same group (16.83 ± 1.95) when compared to the normal group (12.2 ± 0.8). In colclusion, cotreatment of pregnant animals at the implantation phase with indomethacin and rofecoxib was able to induce complete reabsorptions of implantation sites, even with the ovulation process normally occuring, characterized by the presence of corpus lutea.

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ANENCEPHALY AND GASTROSCHISIS BOVINE: A GROSS AND MICROSCOPICAL STUDY

Alberto, M.L.V.[1]; Santos, T.C.[1]; Assis Neto, A.C.[1]; Matsumoto, F.S.[1]; Miglino, M.A.[1]; Ambrósio, C.E.[1]; Mancanares, C.F.[1]; Santos, J.M.[2]

[1] Departament of Surgery, College of Veterinary Medicine and Zootechnology, São Paulo University, São Paulo-SP, 05508-270, Brazil. [2] College of Medicine of the ABC, Anhembi Morumbi University, São Paulo-SP, 04546-000, Brazil. miryan.bio@ig.com.br

The congenital malformations is one of the main causes of the embryonic losses during the pregnancy process. It results of interactions of some factors as multifactorial inheritance, chromossomic and genic alterations and environmental agents, being able still to present unknown etiology. The defects of neural tube are directly associated with nutritional and ambient factors. These factors have important role in the appearance of congenital malformations of the central nervous system, for example the anencephaly. In this case the neuroectoderm is affected, resulting absence of fusing between the neural folds and consequently the brain not close, this event is the principal malformation associated with neural tissue. The gastroschisis is a congenital defect of the abdominal ventral wall, occurring extrusion of the abdominal viscera where umbilical cord is preserved. The etiology of this case may be related with exposition and contact with toxic substances in ambient, as agrotoxic. In the present study, we use 10 bovine embryos from slaughterhouse, we analysis 30 bovine embryos (10 – 60 days of gestation) for gross and microscopical description. Anencephaly and gastroschisis were observed in 10 embryos (33%). We observe the presence of nervous tissue mass (brain) in the cranial region of the embryos, resulting of not formation of part of bones of the cranium and not complete closing of cranial neuropore. In abdominal region, we observed complete fusion of the lateral folds of the body did not occur during the formation of the abdominal wall and the liver was projected to outside.

CYTOLOGIC TYPIFICATION OF THE AMNIOTIC FLUID FROM CALVES FORMED BY PIV AND NATURAL FECUNDATION AT THE DELIVERY MOMENT

Moya, C.F.[1]; Prestes, N.C.[1]; Piagentini, M.[1]; Rocha, N.S.[2]

[1]Department of Animal Reproduction and Veterinary Radiology and [2]Department of Veterinary Clinics of the FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil. carlafredrichsen@yahoo.com.br

The objective of this work was to describe the possible differences in cytological pattern of foetal fluid in the delivery moment comparing the obtained results of Nelore calves from in vitro fertilization with Nelore calves from artificial insemination. Forty animals were divided in two groups: group 1, 20 recipient cows pregnant from PIV embryos; group 2: 20 Nelore cows pregnant from natural fertilization. The animals were kept on a pasture area in Avaré, São Paulo, Brazil. The animals received mineral salt, corn silage and ration as food when closer to the delivery, being transferred to an appropriate place to deliver afterwards. All of the delivery were assisted, during the expulsion and after the rupture of the allantochorion the amnion were punctured to collect 20 ml of fluid, which was kept in a plastic tube and stored in a freezer (-18C). The samples, after being thawed, were centrifuged at 4000 rpm, during 06 minutes (Revan Centrifuga Ciclo Cito) in the Department of Veterinary Patology of the FMVZ/UNESP – Botucatu – SP – Brazil. After deposition of this material on the glass slide, was applied the hematoxilin – Shorr stain (Arruda *et al.*, 1976, modified by Oliveira *et al.*, 2000). To conclude the procedure, the glass slides were prepared and observed in an optical microscopy to classify the types of cells (morphology). The cytological typification was based on the tinctorial affinity of the cells. The cell classification was based on the maturation stage. The basal and parabasal cells were not found on the slides of the amniotic fluid samples analyzed. The small intermediate cells (CIP) had its shape as an oval or polygonal form with large cytoplasm and central nucleus. The large intermediate cell (CIG) showed a central nucleus and a bigger nucleus-cytoplasm relation compared to the superficial cells. These were the largest cells found. They showed keratinization with angular edges, having both a picnotic nucleus (CSN) and no nucleus at all. (CSA). The mean percentages in each cell group were: group 1 CIP (1.9%), CIG (7.1%), CSN (33.5%) and CSA (58%); group 2 CIP (2.9%), CIG (6.8%), CSN (30.6%) and CSA (60%). These findings were difficult to be discussed once there was no report about bovine using similar technique. References reporting amniotic fluid cell classification were found in ovine made through the Nile blue sulfate stain (Souza *et al*, 2000) and the hematoxilin- Shorr was greatly used in the Human Medicine to characterize the epidermal maturity of the fetus in the latter part of pregnancy (Cunha *et al.*, 1978). The mean values found in both groups of this experiment were similar for the four cell types.

BIOCHEMICAL ANALYSE OF AMNIOTIC FLUID OF NELORE CALVES FROM PIV AND NATURAL FERTILIZATION COLLECTED AT DELIVERY MOMENT

Piagentini, M.[1]; Prestes, N.C.[1]; Moya, C.F.[1]; Barretos, C.S.[1]; Lucidi, C.A.[2]; Takahira, R.K.[2]

[1] Department of Animal Reproduction and Veterinary Radiology and
[2] Department of Veterinary Clinics - FMVZ/UNESP, Botucatu-SP, 18618-000, Brazil.
nereu@fmvz.unesp.br

The objective of this study was to evaluate the biochemical composition of the foetal fluids of calves from in vitro production and artificial insemination (AI) in an attempt to correlate the possible changes in the loss of newborn calves in PIV. Forty animals were used. The group 1: 20 cows or heifers (Nelore) with calves from natural fertilization (AI) and the group 2: 20 pregnant cows having PIV calves were fed by pasture and mineral salt, receiving supplement of corn silage and ration when near to the delivery in the rural area in Avaré, São Paulo, Brazil. All of the deliveries were assisted. During the expulsion and after the rupture of the allantochorion was made the puncture of amniotic bag to collect 20 ml of the fluid. The samples were stored at plastic tubes in freezers (-18 C). The analyses were made through commercial biochemical kits. The mean values obtained were: group 1 Urea (45.8 mg/dl), creatinine (8.47 mg/dl), glucose(6.75 mg/dl), total protein (0.44 g/dl), gama GT (33.9 UI/L), chloride (88.41 mmol/L), sodium (82.17 mmol/L), potassium (7.35 mmol/L) and in group 2 Urea (44.1 mg/dl), creatinine (8.44 mg/dl), glucose (5.30 mg/dl), total protein (0.39 g/dl), gama GT (36.50 UI/L), chloride (81.45 mmol/L), sodium (115.5 mmol/L), and potassium (6.3 mmol/L). Both groups 1 and group 2 Urea, Creatinine and Glucose values were discrepant when compared with the values obtained by Li *et al.* (2005). As to the total protein, chloride, sodium and potassium values were similar with the data obtained in the same research group. Baetz *et al*. (1976) obtained almost the similar results as the group 1, regarding the analyzed variables. To the gama GT, no relevant data were found in bovine. However, there is a work regarding Ovine (Prestes *et al.*, 2000) where the value of mentioned variable is practically half the one found in this work. In this experiment, group 1 and 2 showed similar mean values in all evaluated variants.

EARLY IDENTIFICATION OF FETAL SEX AND DETERMINATION OF THE GENITAL TUBERCLE MIGRATION´S DAY IN DAIRY GOATS USING ULTRASOUND

Oliveira, M.A.L.[1]; Santos, M.H.B.[2]; Moraes, E.P.B.X.[2]; Moura, R.T.D.[2]; Chiamenti, A.[2]; Bezerra, F.Q.G.[2]; Rabelo, M.C.[3]; Lima, P.F.[1]

[1]Departamento de Medicina Veterinária/UFRPE (maloufrpe@uol.com.br); [2]Programa de Pós-Graduação em Ciência Veterinária/UFRPE;
[3]Curso de Graduação em Medicina Veterinária/UFRPE

The objective of this study was to identify precociously the fetal sex and determine the day of genital tubercle (GT) migration using ultrasound in Saanen (n = 56) and American Alpine (n = 82) goats. In all the experiments (EI, EII, EIII, EIV), conduced in different periods and properties, the sexing was performed taking into consideration the localization of the GT and identification of penis, prepuce, scrotal bag, nipples and genital swelling. The examinations were carried out with an ultrasound equipped with a linear transducer by transrectal aproach (6.0 and 8.0 MHz). In EI the American Alpine females (n = 24), with 45 to 70 days of gestation, were examined only once. In EII, the American Alpine females (n = 32) were monitored each 12 hours from the 39th to the 57th day of pregnancy. In EIII, the Saanen females (n = 36) with 47 to 77 days of pregnancy were examined only once. In EIV, the Saanen females (n = 46) were monitored each 48 hours, from the 40th to the 58th day of gestation. In EI, there was an accuracy of 85.7% (12/14) in single and 80.0% (16/20) in twin pregnancy, with no significant difference (P > 0.05) between single and twin pregnancies. In EII, the accuracy was 100% (14/14) in single, 87.5% (28/32) in twin and 66.7% (4/6) in triple pregnancy, with a significant statistical difference (P = 0.05) between single and triple pregnancies. In EIII, the accuracy was 100% (24/24) in single, 72.7% (16/22) in twin and 66.7% (2/3) in triple pregnancy. The accuracy of diagnose in single pregnancy was higher (P = 0.05) than in twin and triple pregnancy. In EIV, there was an accuracy of 100% (20/20) in single, 80.9% (34/42) in twin, 66.7% (8/12) in triple and 50.0% (2/4) in quadruple pregnancy. The diagnose accuracy in females with single pregnancy was higher (P = 0.05) than in females with twin, triple and quadruple pregnancy. The total accuracy of diagnosis was 86.6% (26/34) in EI, 88.5% (46/52) in EII, 85.7% (42/49) in EIII and 82.0% (64/78) in EIV, being do not observed difference (P > 0.05) between the experiments. The GT migration of the American Alpine fetuses happens in average on the 46.4 ± 2.1 day of pregnancy and in Saanen on day 48.9 ± 1.8. The results showed that ultrasonography in real time is an efficient method to precociously identify the fetal sex and determine the day of GT migration as well as that repeated exams not always increase the accuracy of fetal sexing.

QUANTITATIVE EVALUATION OF PROTEINS IN UTERINE FLUID OBTAINED DURING THE BOVINE ESTROUS CYCLE

Freire, A.F.[1]; Lima, M.C.[2]; Hucke, E.E.T.S.[3]; Marques, V.B.[2]; Miglino, M.A.[2]; Binelli, M.[1]

[1] Departamento de Reprodução Animal - Av. Duque de Caxias Norte, 225 - FMVZ/USP - Pirassununga, SP, 13635-900, Brasil. anfrevet@usp.br
[2] Departamento de Cirurgia –Setor de Anatomia dos Animais Domésticos e Silvestres - Av. Prof. Dr. Orlando Marques de Paiva, 87 –FMVZ/USP, São Paulo -SP, 05508-900, Brasil.
[3]Laboratório de Fisiologia e Farmacologia - Faculdade de Medicina Veterinária – UNIFEOB, São João da Boa Vista-SP, 13874-148, Brasil.

During bovine early pregnancy, successful embryo-maternal communication from day 15 to 19 after estrous results in luteolysis blocking. However, there is an average 30% failure in this mechanism, leading to embryo death and economic losses. Successful establishment of pregnancy depends on endometrium and conceptus secretory proteins. For the understanding of paracrine mechanisms involved in maintenance of pregnancy it is necessary to obtain and characterize such proteins. Thus, the aim of the present work is to obtain uterine washings during the estrous cycle in cows to (1) evaluate recovery rates of washings, (2) record frequency of contaminated washings and (3) quantify protein concentration of washings. Estrous detection was performed continuously in 11 Holstein cyclic, non-lactating, multiparous cows. From day 0 to 23 of the estrous cycle, uterine washings (500 ml ringer solution) were performed through a Foley catheter. For each day of the cycle, three washings were obtained from different animals. Consecutive washings for a single cow were only performed after a minimum 5-day interval. Volume and aspect of recovered washings were registered. The method of Bradford was used for quantification of total protein concentrations. Recovery rate [(volume recovered/volume infused) x 100] and protein concentration were evaluated by ANOVA. Recovery rates ranged from 77 (D6) to 99.7±6,7% (D7), however, no day of estrous cycle effect was observed ($P>0.1$). From a total of 85 uterine washings, only 9.44% were purulent. No effect of cow or day of estrous cycle was observed ($P>0.1$) on total protein concentrations in uterine washings. Mean protein concentration was 245.69±30.02µg/mL and mean total protein concentration in each infusion was 122.8mg. In conclusion, a collection of uterine washings was obtained throughout the estrous cycle. Recovery rates, frequency of contaminations and protein concentration were adequate. Further experiments are necessary to characterize proteins contained in washings by electrophoresis.

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CANNULATION OF BOVINE UTERUS AS A METHOD TO STUDY REGIONAL FLUID COMPONENTS DURING EARLY PREGNANCY

Lima, M.C.[1]; Freire, A.F.[2]; Hucke, E.E.T.S.[3]; Miglino, M.A.[1]; Binelli, M.[2]

[1]Departamento de Cirurgia –Setor de Anatomia dos Animais Domésticos e Silvestres - Av. Prof. Dr. Orlando Marques de Paiva, 87 –FMVZ/USP, São Paulo -SP, 05508-900, Brasil. limamc@usp.br; [2] Departamento de Reprodução Animal - Av. Duque de Caxias Norte, 225 - FMVZ/USP - Pirassununga, SP, 13635-900, Brasil.; [3]Laboratório de Fisiologia e Farmacologia - Faculdade de Medicina Veterinária – UNIFEOB, São João da Boa Vista-SP, 13874-148, Brasil.

Embryo-maternal communication during the first 3 weeks of pregnancy sometimes is not successful. Failure to block luteolysis leads to embryo death and compromises reproductive efficiency. To minimize economic loss, it is important to study the uterine microenvironment, determining proteins that might be present during pregnancy establishment, specially between days 15 to 19 after estrous. Therefore, the objective of the present study was to develop a method to surgically implant a silicon catheter into the uterine lumen to allow uterine fluid collection. This fluid could be used afterwards to evaluate protein composition and function. First, surgical procedures were tested *ex vivo* so that uterine cannulation in isolated fresh reproductive tracts was performed. Each uterine horn was ligated so that fluid infusion and collection could be performed regionally. Silicon catheters (Hpbio®) tested were inserted into the uterus, aided by a metallic rod, and fixed to uterine serosa, so that only the endpoint of the catheter remained into the uterus lumen. A volume of 40 ml of Ringer solution stained with methylene blue was infused into each uterine horn to evaluate potential leaking. Ligatures were efficient to isolate uterine horns and no leakage was detected. Recovery of infused solution was obtained easily. Then, to test surgical procedures *in vivo*, a laparotomy was performed under local anesthesia (lidocaine 2%) on day 2 of estrous cycle so that uterine horns were exposed, ligated and catheteres implanted. Ends of catheters were exteriorized through an incision in the abdominal wall. Ringer solution was infused and collected from days 13 to 23 of estrus cycle. No leakage was observed, since the total volume infused was recovered, demonstrating that cicatricial process and the ligature were adequate to prevent it. The silicon catheter was efficient for *in vivo* infusions because flow was not interrupted. In conclusion, the present method can be used to regionally collect intrauterine fluid.

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IS ETHANOL LUTEOLYTIC IN COWS?

de Barros, F.R.O.[1]; Dias, L.O.P.[1]; Fantini, D.A.[1]; Ayres, G.R.[1]; Marques, V.B.[2]; Miguez, P.H.P.[3]; Bertan, C.M.[1]; Binelli, M.[1]

[1] Department of Animal Reproduction – FMVZ/USP, Pirassununga-SP, 13635-900, Brazil. [2] Department of Surgery – FMVZ/USP, São Paulo-SP, 05508-900, Brazil. [3] Department of Farmacology – Institute of Biosciences /UNESP, Botucatu-SP, 18618-000, Brazil.
flaviarob@gmail.com

Production of prostaglandins can be influenced by ethanol in many species (Rajakrishnan V., J. Nutr. Biochem 2000, 11:509-514). In experiments conducted in the Physiology and Molecular Endocrinology Laboratory of the University of São Paulo, a significant stimulatory effect of ethanol on production of prostaglandin F2α (PGF-2α) was verified (Binelli M. et al., ICAR 2004, p.135). Because PGF-2α is the most important luteolytic agent in bovines, the purpose of the present study was to verify the luteolytic activity of ethanol in cows. In the first experiment, non-lactating Holstein cows had their estrous cycle synchronized and ovulations verified by ultrasonography. On day 16 of the estrous cycle (D16), the animals were submitted to blood collection from the jugular vein by cannulation, ranked according to body weight and divided into three groups. On D17, blood samples were collected of from each animal every 30 minutes for 4 hours to access PGFM plasma concentrations. At experimental hour 2, animals received the following treatments: ethanol group (0,05 mL 100% ethanol/kg of body weight; intravenous infusion; n=5), control group (0,05 mL saline/kg of body weight; intravenous infusion; n=4) or prostaglandin group (150µg of d-cloprostenol; IM; n=4). Daily blood samples were collected between days 16 and 20 of the estrous cycle for measuring concentrations of progesterone (P_4) in plasma. Plasma concentrations of PGFM and P_4 were measured by radioimmunoassay (RIA) e analyzed by least squares ANOVA. There was an acute release of PGFM in response to ethanol comparing to other treatments (P(0,05). However, only cows treated with PGF-2α underwent luteolysis, based on the decrease of P4 plasma concentrations. In the second experiment, cyclic and non-lactating cross-bred beef cows were slaughtered on day 17 of a synchronized estrous cycle. The reproductive tract was immediately taken to the laboratory and the endometrium was dissected under a laminar flow hood. Endometrial explants of horns contralateral to the corpus luteum (CL) were cultured for 4 hours with culture medium containing 0, 0,1, 1, 10 or 100 µL of 100% ethanol/mL of medium. Concentrations of PGF-2α were measured by RIA and analyzed by least squares ANOVA. It was verified that ethanol did not stimulate PGF-2α production by the endometrium. In conclusion, ethanol does not have luteolytic activity in cows and it should not be used as a tool for the control of the estrous cycles in bovines.

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INCIDENCE OF HYDROMETRA AND MUCOMETRA IN GOATS

Moraes, E.P.B.X.[1]; Santos, M.H.B.[1]; Rocha, J.M.[1]; Arruda, I. J.[2];
Lima, P.F.[3]; Oliveira, M.A.L.[3]

[1]Programa de Pós-Graduação em Ciência Veterinária/UFRPE; [2]Curso de Graduação em Medicina Veterinária/UECE; [3]Departamento de Medicina Veterinária/UFRPE (maloufrpe@uol.com.br)

This work aimed to report the incidence of hydrometra and mucometra in goat of Saanen, American Alpine, Anglo-Nubian and Boer breeds submitted to ultrasonographic exams to diagnose early pregnancy. At the monitored properties, the referred exams were made in females that were kept together with bucks and then separated, at least 30 days, from those. The females that were not diagnose as pregnant and that showed liquid or mucous collection in the uterus, forming hyperechoic and considerably thin mobile trabeculae, were again examined after fiftheen days to confirm pathological condition. The case reports in which the uterine collection exhibited anechoic image were diagnosed as hydrometra and in those with visualization of hypoechoic image were diagnosed as mucometra. For this purpose were used an ultrasound model 240 Parus/Pie Medical, equipped with a linear transducer (6.0 e 8.0 MHz) adapted to a PVC support to facilitate the manipulation throw the rectum of the animal, and an micro-convex (5.0 e 7.5 MHz) covered with a plastic protection on its extremity to avoid vaginal contamination. The females with this uterine alterations received 0.5mg of prostagandin and the ones that exhibited estrous on the following 5 days were again treated with the same substance in equal dose on the 11st day after the first administration. The mates were made on estrous detected by teasers, subsequent to what was evidenced after administration of prostaglandin, using bucks with confirmed fertility. In Saanem goats, 17 (56.7%) were pregnant and from the 13 (43.3%) empties, 9 (30%) were with a normal uterus, 3 (10%) with hydrometra and 1 (3.3%) with mucometra. Among the 23 American Alpines, 4 (17.4%) were pregnant and from the 19 (82,6%) empties, 16 (69.6%) exhibited a normal uterus and 3 (13.0%) hydrometra. In the 34 Anglo-Nubians, 23 (67.6%) were pregnants and from the 11 (32.3%) empties, 10 (29.4%) showed a normal uterus and 1 (2.9%) was with hydrometra. In the 37 Boer goats, 8 (21.6%) were pregnant and 29 (78.4%) empties, among those 27 (74%) showed a normal uterus, 1 (2.7%) exhibited hydrometra and 1 (2.7%) mucometra. All the females treated with prostaglandin exhibited estrous, being 3 (75.0%) Saanen, 2 (66.0%) American Alpine, 1 (100%) Anglo-Nubian and 2 (100%) Boer, after the first administration, and 1 (25.0%) Saanen and 1 (34%) American Alpine after the second administration. Between the 30th and the 35th day of the mate, the females treated were examined by ultrasound and 10 (100%) were diagnosed as pregnant. The results did not show difference (P = 0.05) in the incidence of hydrometra and mucometra among the monitored herds, allowing to conclude that ultrasonography is an important tools to detect both early pregnancy and uterine alterations, as well as to help handling small ruminants.

EFFECT OF UTERINE WASHINGS ON DURATION OF THE ESTROUS CYCLE IN CATTLE

Binelli, M.[1]; Freire, A.F.[1]; Lima, M.C.[2]; Hucke, E.E.T.S.[3]; Marques, V.B.[2]; Miglino, M.A.[2]

[1] Universidade de São Paulo - Faculdade de Medicina Veterinária e Zootecnia - Departamento de Reprodução Animal - binelli@usp.br / anfrevet@usp.br - Av. Duque de Caxias Norte, 225 - FMVZ-VRA - Pirassununga, SP, Brasil - 13635-900; [2] Universidade de São Paulo - Faculdade de Medicina Veterinária e Zootecnia - Departamento de Cirurgia –Setor de Anatomia dos Animais Domésticos e Silvestres - limamc@usp.br / miglino@usp.br / vamarkes@usp.br - Av. Prof. Dr. Orlando Marques de Paiva, 87 -FMVZ - VCI- São Paulo -SP, Brasil - 05508-900; [3]Centro Universitário da Fundação de Ensino Octavio Bastos UNIFEOB - Laboratório de Fisiologia e Farmacologia - Faculdade de Medicina Veterinária – São João da Boa Vista-SP, Brasil – ehucke@feob.br – 13874-148

During bovine early pregnancy, successful embryo-maternal communication from day 15 to 19 after estrous results in luteolysis blocking. However, there is an average 30% failure in this mechanism, leading to embryo death and economic losses. Successful establishment of pregnancy depends on endometrium and conceptus secretory proteins. For the understanding of paracrine mechanisms involved in maintenance of pregnancy it is necessary to obtain and characterize such proteins. On way of obtaining uterine secretions is through uterine washings using a Foley catheter. However, executing washings may alter uterine physiology and affect duration of estrous cycles. Thus, the aim of the present work was to evaluate the effect of performing uterine washings on duration of the estrous cycle in cows. Estrus detection was performed twice daily, for 60 minutes, in 11 Holstein cyclic, non-lactating, multiparous cows for a period of 180 days. From day 0 to 23 of the estrous cycle, uterine washings (500 ml ringer solution) were performed through a Foley catheter. For each day of the cycle, three washings were obtained from different animals. Consecutive washings for a single cow were only performed after a minimum 5-day interval. From the 66 estrous cycles examined, 9.1% had 18 days or less, 83.3% had between 19 and 23 days and 7.6% had 24 days or more. In conclusion, with the uterine washing protocol used, it was possible to store a collection of washings obtained throughout the estrous cycle without altering the frequency of cycles with normal duration. Further experiments are necessary to characterize proteins contained in washings by electrophoresis.

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HISTOPHATOLOGICAL ASPECTS OF ADENOMYOSIS IN BOVINE UTERUS IN DIFFERENT PHASES OF ESTROUS CYCLE

Moreira, L.[1]; Caldas-Bussiere, M.C.[2]; Quirino, C.R.[2]; Carvalho, E.C.Q.[1]

[1]Morphology and Pathological Anatomy Sector-LSA/CCTA/UENF. [2]Animal Reproduction Sector-LMGA/CCTA/UENF, Av. Alberto Lamego, 2000, Campos of Goytacazes-RJ, 28013-620, Brazil. moreira@uenf.br

Uterine Adenomyosis is the term used to designate distopic localization of endometrial glands and stroma within sheaves of the myometrium. Studies suggest that adenomyosis has as primary cause the disruption of the endometrium-myometrium border by unbalance among the sexual hormones, for successive pregnancy terminations and advanced age. In women and laboratory's animals it is a very defined finding and it is associated with sub-fertility and infertility. During the estrus in cows, the endometrial glands are relatively straight and as the progesterone concentration increases, the glands become more coiled, and the maximum of grow up/coiled happens about the eighth day following ovulation. The epithelial surface is relatively tall during the estrus, cuboidal at two days of proestrus and it reaches a maximum height at nine to twelve days of the cycle. This present investigation had the intention to connect characteristics of the adenomyosis with the phases of the estrous cycle. The uterine pieces from 61 cows were collected in slaughterhouses at Campos, RJ. The samples were fixed in tamponed formalin neutral buffered at 10%, submitted to routine histotechnique and staining by hematoxilin/eosin. A score was idealizes in order to help at microscopy, where the adenomyosis was classified as superficial and deep. The morphology, staining and vascularization of the corpus luteum and the presence or not of ovarian follicles larger than 8mm estimated the phase of the estrous cycle. The data were analyzed by the qui-square test (α=0.05). The cows in anestrus (n=11) showed smallest adenomyosis percentage of occurrence (8.2%), while the animals in the medium luteal phase (n=21) exhibited the largest percentage (31%). The initial luteal (n=13) and follicular phase (n=16) exhibited similar percentages of adenomiose occurrence (18.03 and 22.9%, respectively). The largest percentage of deep adenomyosis happened in the initial and medium luteal phase (45 and 47.4%, respectively) when compared with the percentage during the anestrus (20%) and during the follicular phase (14.3%). These data suggest that the cycle phase influences in the adenomyosis occurrence and in the degree of infiltration of the endometrial glands.

SEX STEROIDS MODULATE THE SYNTHESIS PGF2 ALPHA IN BOVINE ENDOMETRIAL EXPLANTS

Miguez, P.H.P.[1]; Da Cunha, P.M.[2]; Marques, V.B.[3]; Bertan, C.M.[2]; Binelli, M.[2]

[1] Department of Pharmacology IB/UNESP, Botucatu-SP, 18618-000, Brazil.; [2] Department of Animal Reproduction FMVZ/USP, Pirassununga-SP, 13635-900, Brazil. [3]Department of Surgery FMVZ/USP, São Paulo-SP, 05508-900, Brazil. phmiguez@yahoo.com.br

In cattle, the luteolisys is caused by pulses of endometrial prostaglandin F2α (PGF2α) at the end of the luteal phase of the estrous cycle. The estradiol (E_2) and the Progesterone (P_4) are important to initiate luteolysis in ruminants, but mechanisms of action hormones are unclear. General objective of this paper was to investigate the effects of E_2 and P4 alone or in combination on PGF2α production in cattle. Were used cross-bred beef cows abated in D17 of the estrous cycle synchronized in three independent studies. The endometrium was dissected and the endometrial explants were placed in culture plates and incubated in the presence of several treatments in triplicate. The concentrations of PGF2α in culture plates were quantified by radioimunoensay**.** Study 1, was conducted to test the capacity of E_2 to stimulate synthesis of PGF2α in bovine endometrial explants from three cows in the concentrations 0, 10^{-12} or 10^{-11}M. It was observed that the E_2 was not capable of stimulated the synthesis of PGF2α (P>0.1). Study 2, was conducted to test the capacity of P4 to stimulate synthesis of PGF2α in bovine endometrial explants from four cows in the concentrations 0, 10^{9}, 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M. Observed P_4 was not capable of stimulated the synthesis of PGF2α (P>0,1). Study 3, Tested the interaction between P_4 e E_2 in the stimulation synthesis of PGF2α in bovine endometrial explants of three cows. The edometrial explants received P_4 (0,10^{-8} or 10^{-7}M), E_2 (0,10^{-12}, 10^{-11}M) or combination of this concentration of P_4 and E_2. Had stimulate in production of PGF2α (P<0,01) when associate with P_4 and E_2 and the following concentrations: 10^{-11}M E_2 + 10^{-8}M P_4, 10^{-11}M E_2+10^{-7}M P_4. In conclusion, synthesis of PGF2α requires an interaction between E2 and P4 to have a stimulus in the synthesis of PGF2α. However, molecules mechanisms involved in this **in**teraction are not understood.

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MODULATION OF PROSTAGLANDIN F2α PRODUCTION BY SERUM FACTORS IN CATTLE

Dias, L.O.P.[1]; de Barros, F.R.O.[1]; Fantini, D.A.[1]; Meneghini, R.C.M.[1]; Pontes, E.O.[1]; Bertan, C.M.[1]; Miguez, P.H.P.[2]; Binelli, M.[1]

[1] Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Duque de Caxias Norte 225, 13630-000 Pirassununga-SP, Brazil. pontes.eduardo@uol.com.br [2] Departamento de Farmacologia, Instituto de Biociência, Universidade Estadual Paulista, Distr. Rubião Jr. s/n, 18618-000 Botucatu-SP, Brazil

Earlier studies reported an increase in the spontaneous release rate of prostaglandin F2α (PGF2α) by cultured bovine endometrial explants. In contrast, the production observed *in vivo* is constant. The hypotesishypothesis of the present paper was that serum factors, absent *in vitro,* modulate the production of PGF2α. The objectives were to test the effect of bovine serum (Experiment 1) and serum P_4 in the modulation of PGF2α release (Experiments 2 and 3). A serum pool of five cows on day 17 of the estrous cycle was prepared and used in the experiments. Non-lactating, crossbred cows were synchronized and slaughtered on day 17 of the estrous cycle. Reproductive tracts were taken to the laboratory immediately after slaughtering. Endometrium from ipsilateral horns were dissected and explants (~50mg) were conditioned in culture plates containing culture medium (Ham-F10). In Experiment 1, endometrial explants from three cows were cultured in medium containing 0, 20 or 40% serum. In Experiment 2, explants **were cultured in medium supplemented with 0 or 40% serum, depleted of steroids and conditioned with 0 or** 4ng/ml **of** P_4. In Experiment 3, explants were cultured in medium supplemented with 0 or 40% of serum containing 0 or 10^{-6} M **mifepristone (RU-486).** The tissues were incubated in an orbital shaker at 37º C for 15 hours and medium samples collected after 1, 8 and 15 h. Concentrations of PGF2α were measured by radioimmunoassay. In Experiment 1, production of PGF2α decreased as concentration of serum in medium increased ($P<0.01$). In Experiment 2, addition of serum inhibited the production of PGF2α (serum effect; $P<0.01$) and such effect was independent of the presence of P_4 in the medium (P_4 effect and serum x P_4 interaction effect; $P>0,1$). In Experiment 3 the addition of serum inhibited the production of PGF2α (serum effect; $P<0.01$) and such effect was not influenced by the blocking P_4 receptors with antagonist RU-486 (RU-486 effect and serum x RU-486 interaction effect; $P>0.1$). It was concluded that serum factors in bovine serum are involved in the modulation of PGF2α release, but P_4 does not participate of this modulation.

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QUANTITATIVE INHIBITION OF PROSTAGLANDIN-F2A SYNTHESIS: BASIS FOR A BIOASSAY TO MEASURE BOVINE INTERFERON-TAU ACTIVITY

Marques, V.M.[1]; Bertan C.M.[2]; Merighe G.K.F.[3]; Meirelles F.V.[3]; Miglino, M.A.[1]; Binelli, M.[2]

[1] Universidade de São Paulo - Faculdade de Medicina Veterinária e Zootecnia - Departamento de Cirurgia setor Anatomia dos Animais Domésticos e Silvestres - vamarkes@usp.br / limamc@usp.br / miglino@usp.br - Av. Prof. Dr. Orlando Marques de Paiva, 87 FMVZ - CBRA - São Paulo -SP, Brasil - 05508-900; [2]Universidade de São Paulo - Faculdade de Medicina Veterinária e Zootecnia - Departamento de Reprodução Animal - Pirassununga, SP - Brasil - binelli@usp.br / anfrevet@usp.br / cbertan@fmvz.usp.br - Av. Duque de Caxias Norte, 225 - Pirassununga, SP, Brasil – 13630-000; [3]Universidade de São Paulo - Faculdade Zootecnia e Engenharia de Alimentos - Departamento de Ciências Básicas - Pirassununga, SP - Brasil - Av. Duque de Caxias Norte, 225 - Pirassununga, SP, Brasil – 13630-000

In cattle, maternal recognition of pregnancy is mediated through interferon-tau (IFN-t). IFN-t is a conceptus secretory protein which main action is to block production of prostaglandin-F2α (PGF2α) in the endometrium. Activity of IFN-t is usually measured indirectly, via an antiviral assay. It is proposed that a more adequate assay should measure IFN-t's antiluteolytic activity, which is the ability to inhibit production of PGF. Objective was to verify whether IFN-t could quantitatively inhibit phorbol-ester induced synthesis of PGF2α in a line of bovine endometrial cells (BEND cells). In Experiment 1, BEND cells were platted on 48-well plates and treated in triplicate with 0, 6.25, 12.5, 25, 50, 100 or 200 ng/ml of 12, 13-phorbol dibutyrate (PDBu) for 6 h in three independent repetitions. Concentrations of PGF2α in culture medium were measured by radioimmunoassay. The PDBu stimulated PGF synthesis in a concentration-dependent manner ($P<0.01$). Twenty five ng/ml PDBu elicited sub-maximal synthesis of PGF2α. In Experiment 2, BEND cells were treated in triplicate with 25 ng/ml PDBu associated with 0, 0.2, 0.4, 0.8, 1.6, 3.2 or 6.4 ng/mL recombinant bovine IFN-t for 6 h in four independent repetitions. The IFN inhibited PDBU-stimulated synthesis of PGF2α in a concentration-dependent manner ($P<0.01$). Percent inhibition of PGF2α synthesis was associated positively and linearly to concentration of IFN-t ($R^2=0.87$; $P<0.01$). Calculated concentration of IFN which caused 50% inhibition was 5.2 ng/ml. It was concluded that IFN-t has the ability to quantitatively inhibit PDBu-induced synthesis of PGF2α. It is expected that the antiluteolytic activity in biological fluids of interest can be measured using a similar assay system.

EFFECT OF DIFFERENT PROGESTERONE CONCENTRATIONS DURING THE ESTROUS CYCLE IN ESTRADIOL 17β–INDUCED PROSTAGLANDIN-F2(PRODUCTION IN CATTLE

Fantini, D.A.1; Goissis, M.D.1; Barros, F.R.O.1; Dias, L.O.P.1; Bressan, F.F.1; Miguez, P.H.P.[2;] Bertan, C.M.1; Marques, V.B.3; Almeida, A.B.1; Binelli, M.1.

[1] Department of Animal Reproduction – FMVZ-CBRA/USP, Pirassununga-SP, 13635-900, Brazil.
[2]Department of Farmacology – Institute of Bioscience/UNESP, Botucatu-SP, 18618-000, Brazil.
[3]Department of Surgery – FMVZ/USP, São Paulo-SP, 05508-900, Brazil. dafantini@yahoo.com.br

Prostaglandin F2α (PGF2α) has an important role in bovine luteolysis. PGF2α production is regulated by a coordinated action of progesterone (P4) and estradiol (E2). It is well know that E2 injections can stimulate PGF2α release and this can be modulated by P4. It is possible that maintenance of pregnancy is mediated through an action of P4 to block PGF2α production and luteolysis. Objective of this study was to verify whether E2-induced PGF2α production differs in cows with different concentrations of plasma P4 during the estrous cycle. Non lactating Holstein cows, on day 6 (D6) of a synchronized estrous cycle (OvSynch), were treated with 0 (control group; n=9) or 5000 IU im (hCG group; n=8) of human chorionic gonadotropin (hCG; Vetecor®), to stimulate formation of an accessory corpus luteum (CL). On D8, accessory CL formation was confirmed by ovarian ultrassonography. Blood samples were collected from D6 until estrous manifestation for P_4 measurement. On D17, all cows were jugular cannulated, and blood samples were collected for 12 hours at every 30 minutes (experimental hours – 2 to 10) for 13,14 dihydro 15-keto PGF2α (PGFM) measurement. At experimental hour 0, 3 mg iv of E2 were administered to all animals. Serum concentrations of P4 and PGFM were measured by radioimmunoassay and were analyzed by least squares ANOVA. There was no difference of mean serum concentration of P_4 along the estrous cycle ($P>0.1$). However, there was a greater mean P_4 production in hCG group than in control group on D11, D14 and D18. There was a greater mean serum PGFM production in hCG group between experimental hours 4 and 5.5 ($p<0.05$), with a variation of 14.77 up to 85.52 pg/ml. Considering that P_4 has a role in stimulating an E2-induced PGF2α production, it was concluded that the P4 action on pregnancy maintenance is to stimulate conceptus growth, and not to block PGF2α synthesis.

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EFFECT OF THE USE OF D-CLOPROSTENOL AND OCITOCIN IN THE UTERINE INVOLTION OF DAIRY COWS

Vasconcelos, R.S.[1]; Rigo, A.G.[1]; Lisboa, L.A.[1]; Ribeiro, A.B.C.[2]; Sgobero Filho, I.[3]; Chacur, M.G.M.[4]; Seneda, M.M.[1]

[1] Departament of Veterinary Clinic – UEL/PR, Brazil. [2] Sítio Santana – Londrina-PR, Brazil. [3] Produtiva Ltda – Londrina-PR, Brazil.[4] Unoeste – Presidente Prudente-SP, Brazil.
rschroeder@pop.com.br

The condition of the uterus in post-partum has direct influence in the fertility of the dairy cows and in the production of milk.The normal uterine involution is essential to return to the reproductive phase. This study has the objective of evaluating the effect of ocitocin and prostaglandin in post-partum uterine involution in high-yielding dairy cows. The experiment was carried out in a farm located next to Londrina-PR. Fifty-nine highly productive dairy cows were used in the experiment, all of them of the Holstein breed, with average body score 3.5, following a table of 1 to 5 and with average weight 654.4Kg. Only cows with eutocic delivery had been considered in the experiment. The animal had been randomly divided in 3 groups. In the control group (n = 17) no treatment was made, in group 2 (n = 18) a dose of ocitocin (Orastina Forte®, 30 UI, IM) was administered at the moment of delivery and another dose 4 hours after the first one. In group 3 (n = 24) a dose of cloprostenol (Preloban®, 0.15mg, IM) was administered at the moment of delivery and another dose 3 days after the first one. The uterine involution was evaluated by rectal palpation, approximately 30 days after delivery. The criteria adopted for the degree of uterine involution were: G1 - excellent, complete involution of the horns; G2 - satisfactory, partial involution of the horns and G3 - unsatisfactory, presence of content in the uterus. The control group presented 41.2% (7/17) of the animals classified as G1, 47% (8/17) as G2 and 11.8% (2/17) as G3; the ocitocin group presented 27.8% (5/18) as G1, 61.2% (11/18) as G2 and 11% (2/18) as G3; and the group treated with prostaglandin had 54.2% (13/24) as G1, 29.1% (7/24) as G2 and 16.7 (4/24) as G3. In relation to the results, the use of prostaglandin at the moment of birth followed by a new dose 3 days after, was the most promissing treatement for uterine involution. Although this paper had a preliminary character the data obtained is in accordance to the current literature, when refering to the efficiency of prostaglandin in uterine involution.

RELATIONSHIP OF THE INTRAUTERINE NITRITE CONCENTRATION WITH THE OCCURRENCE OF UTERINE ILLNESSES IN BOVINE

Moreira, L.[1]; Viana, K.S.[2]; Caldas-Bussiere, M.C.[2]; Quirino, C.R.[2]; Carvalho, E.C.Q.[1]

[1]Morphology and Pathological Anatomy Sector - LSA/CCTA/UENF. [2]Animal Reproduction Sector - LMGA/CCTA/UENF, Av. Alberto Lamego, 2000, Campos of Goytacazes-RJ, 28013-620, Brazil. moreira@uenf.br

In the beef livestock, females that showed reproductive inefficiency, they are usually discarded without a more detailed analysis of your reproductive report. The nitric oxide (NO) is synthesized by nitric oxide synthase from the amino acid non- essential L-arginine in NO and L-citruline. The NO is quickly rusted at nitrite/nitrate by cells as defense mechanism, mainly when produced in high concentration. Besides, the NO has been implicated as one of the effectors of the mice early embryonic lost. The present study had as objective relates the nitrite concentration in the cow's uterine lumen attacked by uterine pathologies (adenomyosis, endometritis, cystic endometrial hyperplasic (CEH), angiomatosis dystrophy and periglandular fibrosis) or not. Uterine pieces were collected from 25 unpregnant cows in slaughterhouses of Campos, RJ. The intra-uterine washed with 1ml of PBS was accomplished in order to measure of the nitrite concentration by the Griess reaction. The uterine horn, which the wash was accomplished, it was submitted to histopathology for diagnosis of the referred pathologies. The data were analyzed by ANOVA and *t* test (α=0.05). A significant relationship with periglandular fibrosis (99.15±89.75mM), endometritis (102.96±87.58mM) and angiomatosis dystrophy (157.57±108.80mM), and not significant with the adenomiose (77.40±81.25mM), CEH (73.94±43.41mM) and normal uteruses (15.29± 20.85 mM) for the variance analysis and F test it was verified. However, high nitrite concentration showed a significant relationship with accentuated adenomyosis superficial and deep for the t test. In the observation of the individual concentration of nitrite made to think us there is a cumulative effect in the NO generation, because when we analyze the data separately, the nitrite concentrations largest (281.14; 265.36; 105.33 and 117.82 mM) it presented at least two pathologies happening simultaneously in the same analyzed uterine segment. These results show that there is a positive relationship with type and amount of uterine pathologies with nitrite concentration in uterine lumen. More studies are necessary to verify the high concentration of NO in these pathologies and it would be one of the reasons of the cows sub-fertility or infertility.

EFFECT OF GESTAGENS ON THE EXPRESSION OF STEROID RECEPTORS ON SHORT-LIFE CORPUS LUTEUM AND ENDOMETRIUM IN POSTPARTUM ANESTROUS COWS

Loguercio, R.S.[1]; Pimentel, C.A.[2]; Loguercio, L.P.[3]; Löf, H.K.[1]; Fernandes, R.[1]; Oliveira, J.F.C.[1]; Gonçalves, P.B.D.[1]

[1] Universidade Federal de Santa Maria, Departamento de Clínica de Grandes Animais Laboratório de Biotecnologia e Reprodução Animal - BioRep, Santa Maria, RS, Brasil. bayard@biorep.ufsm.br; [2] Departamento de Patologia Animal – UFPel, Pelotas- RS – Brasil
[3] Granjas 4 Irmãos S&A – Rio Grande – RS – Brasil.

The aim of the present study was to evaluate the effect of gestagens on the expression of progesterone receptor (PR) and estrogen alpha (ERα) and beta (ERβ) receptors in the endometrium and CL, to elucidate part of the early mechanism of luteolysis during the first postpartum luteinization (short-life CL). Ten anestrous cows with 30 days postpartum were divided into two groups. In the anestrous group (AN; n=5), the follicular wave of each cow was monitored daily. When the dominant follicle reached a diameter of 12 mm, gonadorelin (GnRH agonist; 100 μg; im) was injected and luteal and endometrial tissues were collected 7 days later for the gene expression (RT-PCR) and histological analyses. In the AN+MAP (n=5) group, the animals received medroxyprogesterone acetate (MAP; 250 mg; vaginal device) for 7 days and estradiol benzoate (5 mg; im) at insertion of the devices. In this group, the evaluation of follicular wave, injection of GnRH and collection of sample tissue were similar to the AN group. The control group consisted of 10 cycling cows designated to the abattoir where samples of endometrium and corpus luteum were collected. The collected samples were submitted to the analysis of gene expression (RT-PCR) and histological evaluation. Pre-exposure to gestagen during the first postpartum estrous cycle influenced the expression of steroid receptors in the endometrium. The PR expression was higher in the endometrium of the AN+MAP group animals compared to the other groups ($P<0.05$), suggesting an inhibitory action of gestagen at the beginning of the luteolytic cascade. The expression of ERα was higher in the endometrium of animals from the AN and AN+MAP groups compared to cycling animals ($P<0.05$). Animals from the AN+MAP group did not express ERβ in endometrial tissue, in contrast to the AN group and cycling animals ($P=0.0834$). In the AN group, the animals presented higher REβ expression in the CL($P=0.0352$), suggesting a regression of the CL. The endometrial glandular epithelium was higher and the number of large luteal cells were greater in animals previously exposed to gestagen (AN+MAP) than in the AN group ($P<0.05$). These findings indicate that the exposure to gestagen prior to the first postpartum estrous cycle results in physiological changes similar to the normal estrous cycles. Also, there are strong evidences that the steroid receptors play an important role in the mechanisms of premature luteolysis during the end of the postpartum anestrous in the cow.

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ANALYSIS OF +15 REGION OF α-LACTALBUMIN GENE IN NELORE CATTLE (*Bos primigenius indicus*)

Martins, L.F.; Milazzotto, M.P.; Feitosa, W.B.; Mendes, C.M.; Nicacio, A.C.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
leydson_martins@yahoo.com.br

The α-lactalbumin is a subunit of lactose synthase, that is responsible for junction of glucose and galactose to form lactose, that is responsible for milk yield. The A_{+15} allele is related to higher milk yield than B_{+15} allele in Holstein cattle. The aim of this work was to analyze the polymorphism in +15 region of a-lactalbumin gene in Nelore cattle, to improve genetic programs and animal transgenesis. Two animal groups were used, one control with 15 Holstein cattle and one experimental with 50 Nelore cattle. The genomic DNA was extracted from peripheral blood by the Phenol/Cloroform method and amplified with especific started oligonucleotide, resulting in one fragment of 167 bp. The amplified sample was incubate with *MnlI* enzime at 37ºC for 16 hours. After the digestion, samples was analyzed in poliacrilamide gel 12% and three alleles were identified: A_{+15} that resulted in three fragments 77, 50 and 40 bp and B_{+15} with two fragments 117 and 50 bp, both already described in literature and a third new allele, named C, that resulted in two fragments 137 and 30 bp. The frequency was 20% of AB and 80% of BB in control group animals and 10% of BB and 90% of BC in experimental group. In conclusion, results showed predominance of alelle B_{+15} in the two groups and presence of A_{+15} allele only in Holstein and allele C_{+15} only in Nelore cattle.

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STUDY OF -1689 REGION OF α-LACTALBUMIN GENE IN NELORE CATTLE (*Bos primigenius indicus*)

Martins, L.F.; Milazzotto, M.P.; Caetano, H.V.A.; Feitosa, W.B.; Yamada, C.; Assumpção, M.E.O.A.; Visintin, J.A.

Department of Animal Reproduction – FMVZ-USP, São Paulo-SP, Brazil
leydson_martins@yahoo.com.br

The milk yield is directly proportional to lactose, a dissacarid composed of glucose and galactose. The lactose synthase is the enzime responsible for this sugars binding and it has two subunits named galactosil/ transferase and a-lactalbumin. Voelker el al. (Journal Dairy, v.80, 9, 194-197, 1997) described in – 1689 position, by RFLP. Swedish red and white breed has a correlation between polymorphism at this region and milk lactose concentration. Animal –1689 AA with +15 BB genotype have 0,08% more lactose per milk kg than animals –1689 AB with +15 BB genotype. The aim of this work was to analyze the polymorphism in the – 1689 region to the a-lactalbumin gene in Nelore cattle, to improve genetic programs and animal transgenesis. Two groups were used, one control with 15 Holstein cattle and one experimental group with 50 Nelore cattle. The genomic DNA was extracted from periferic blood by the Phenol/Cloroform method and amplified with especific started oligonucleotide. The amplified samples were incubated with *SduI* enzime at 37ºC for 16 hours. After digestion the sample was analyzed in polyacrilamide gel 12%, showing two alleles: A_{-1689} allele no digested and B_{-1689} allele with two fragments 260 and 169 bp. The frequency was 33% of animals AA, 60% AB, and 7% BB in control group and 100% AA in experimental group. In conclusion, althogh Nelore breed have the genotype for higher milk lactose production is necessary to consider the analyses and alelle correlation of +15 region of a-lactalbumin gene to select animals of interest.

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DIMETHYL FORMAMIDE AND COCONUT WATER IN CANINE SEMEN CRYOPRESERVATION

Zimmermann, M.[1]; dos Santos, T.E.[1]; Blume, H.[1]; Snel-Oliveira, M.V.[1]; Fidellis, A.A.G.[1]; Mondadori, R.G.[1]

[1]Animal Reproduction Laboratory, Veterinary Medicine Departament, UPIS – Faculdades Integradas, SEPS EQ 712/912, Conjunto A, Brasília – DF, 70390-125. mondadori@upis.br

The aim of the present study was to evaluate de efficacy of different extenders and cryoprotectants in canine semen cryopreservation. The semen pool used in the experiment was obtained from de second fraction of the semen collected by digital manipulation from three mixed breed dogs aged from four to seven years old, maintained at UPIS University. The semen collection was performed once a week, during six weeks. Each week corresponds to one experiment repetition. The semen pool was divided in five fractions in order to be extended in one of the following freezing media: (1) tris-egg yolk with 6% of glycerol (**TEY6G**); tris-egg yolk with 3.5% of dimethyl formamide (**TEY3.5DF**); tris-egg yolk with 7% of dimethyl formamide (**TEY7DF**); tris-egg yolk with 14% of dimethyl formamide (**TEY14DF**) and; coconut water-egg yolk with 6% of glycerol (**CWEY6G**). Following de initial dilution (extender without cryoprotectant) the semen aliquots was chilled for 90 minutes until 4ºC, after this period the second part of the extender (with cryoprotectant) was added and the whole mixture was charged in 0.5mL straws. The straws were placed in liquid nitrogen vapor for 15 minutes, until -60ºC, then they were plunged in liquid nitrogen. The semen progressive motility and vigor were evaluated in three different moments: just after the pool formation, after the dilution and chilling and after thawing. The sperm morphology was evaluated by means of semen smears, obtained previous of pool dilution and after thawing, stained with Congo Red, and analyzed under light microscopy using a 100X objective and immersion oil. To evaluate the results of progressive motility and vigor it was used variance analysis and the means were compared using Tukey test. In order to analyze the significance of the sperm morphology difference, the qui-square test was used. Fresh semen progressive motility and vigor were, respectively, 87% and 5. After chilling the progressive motility showed a medium decrease of 7% ($p<0.05$), without statistical difference between treatments ($p>0.05$). The vigor showed a similar behavior with a decline of 8% among the two periods ($p<0.05$) and statistical difference between the treatments was not observed. When the post-chilling and post-thawing semen vigor were compared, it was not detected statistical difference between treatments, but, the mean vigor reduction (30%) was significant ($p<0,001$). Finally, as the vigor, the comparison of progressive motility post-chilling and post-thawing, detected a significant drop for all the treatments. However, in this case, the treatments which showed a more pronounced drop (73%) were **TEY3.5DF, TEY14DF** and **CWEY6G**, this group of treatments significantly differed from **TEY7DF** (46%), which differed from **TEY6G** treatment, that showed the lower fall (29%). By the analysis of sperm morphology, it was revealed that all the treatments ($p>0.05$) showed a similar increase (22%) in the number of defective cells between the pre-chilling and post-thawing. In conclusion, the results revealed that more studies must be performed, mainly with dimethyl formamide concentrations around 7%, using other methods to verify sperm viability. The coconut water extender must be submitted to other challenges with different cryoprotectans and concentrations.

STANDARDIZATION OF A DOUBLE STAIN USING *TRYPAN BLUE* PLUS *GIEMSA* TO EVALUATE DOG SPERM.

Ribeiro, A.E.[1,2]; Salavessa, C.M.[1,2] ; Cunha, I.C.N.[2]; Paes de Carvalho, C.S.[2]; Silva, J.F.S.[2]

[1]Experiment conducted during under graduation course of author with FENORTE/TECNORTE scholarship and FAPERJ financial support.; [2]Laboratório de Melhoramento Genético Animal/ CCTA/ UENF- Av. Alberto Lamego, 2000. Campos-RJ. cunhaicn@uenf.br

Didion et al., (Gamete Research, v.22, p.p.51-57, 1989) proposed a double stain to bovine species using: trypan blue (TB), to detect spermatozoa (sptz) viability and giemsa (G) to verifyacrossomal status. These authors recommend this test to distinguish between the authentic acrossomal reaction (RA) process in witch the spermatic cell remains intact while the fusion of the plasmatic and extern acrossomal membranes occurs, and the false R.A., where all the spermatic membranes of the sptz are damaged. The objective of the present study is to standardize the trypan blue and giemsa (TBG) methodology to the canine specie. Experiment 1 – four ejaculates were collected from adult dogs and divided in identical aliquots with 100±50µL, in each aliquot TB was added in the proportion of 1:1. These mixtures were than incubated at 37°C for 10 minutes, centrifuged for 6 minutes (3000 rpm) in TALP SP medium, after that the supernatant was discarded. The centrifugation in TALP SP step was performed twice consecutively. Nine smears were prepared with de pellet. Each 3 smears were incubated in G prepared in the following proportions: 1:9, 1:10 and 1:12 (stain:water) and, for each dilution 14, 16 and 18 hours incubation were used. Smears were evaluated under bright-field microscopy (1000x). The incubation with *giemsa* prepared in the proportion of 1:9 with water for 18h was the best stain result obtained and with these protocol the four sptz classes described by Didion et al. (1989) could be observed. Experiment 2 – ten ejaculates of adult dogs with progressive motility =50% were used. Each ejaculate was divided and processed forming two groups: GM- semen sample was submerged in liquid nitrogen and than taken to a water bath at 37°C, consecutively until all sptz presented no movement and GV – sample maintained at 37°C. Smears from both groups (GM and GV) were prepared and stained according to the best results obtained in the Experiment 1. In each smear a total of 200 sptzs were evaluated under bright-field microscopy (1000x) and classified according classes: **dead sptz with intact acrosome** – blue head and pink acrosome; dead sptz with damaged acrosome (**false R.A.**) – blue or purple head with uncolored acrosome; **alive sptz with intact acrosome** – pink head and acrosome and alive sptz with reactive acrosome (**R.A.**) – pink or white head with uncolored acrosome. Concerning the Experiment 2, the GM group (stressed by large temperature variation) presented 97,8% of the sptzs presenting stain characteristic of the false R.A. and the GV group with 92,9% of the sptzs presenting a coloration characteristic of intact sptzs. Therefore, according to the above described results it is possible to conclude that the TBG coloration method described by Didion et al. (1989) could be adapted and successfully used for distinguishing dead and alive sptzs for canine species following the above mentioned modifications.

AUTHORS INDEX

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