Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Effects of 17β-estradiol in the abundance of transcripts for enzymes involved in the synthesis of endometrial PGF2α in bovine females at the end of diestrus

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In bovine females, 17β-estradiol (17β-E2) stimulates the synthesis of endometrial PGF2α, however the mechanisms of this action are unknown.The aim of this study was to determine the effects of 17β-E2 on the abundance of transcripts (PKCα, PKCβ, PLA2G4, AKR1B1, AKR1C4 and PTGS2) involved in the synthesis of PGF2α. Nellore Heifers (N=50) were synchronized by the insertion of intravaginal progesterone (P4) device (0.558g; Cronipress®), estradiol benzoate (1mg; Sincrodiol®) and D-Cloprostenol (0.075mg; Croniben®), both intramuscular (IM). After 6 days, D-Cloprostenol was injected, IM. After 48 hours, the device of P4 was removed and D-Cloprostenol (0.150mg) was administered IM. On this occasion, an adhesive was inserted at the base of the tail for the identification of estrus (Boviflag Red Estrus Detector - ABS Pecplan) and observations of estrus were made in the next 4 days. Only heifers identified in estrus and that ovulated (N=46) participated in the experiment. At D15 (D0 = estrus day) heifers were randomly divided into two groups: Control Group (C; 2mL of pure sesame oil, IM; N=22) or Estradiol (E; 1mg 17β-E2 diluted in 2ml of pure sesame oil, IM, N=24). The time of administration of treatments was considered time zero. Blood samples were obtained from 0h to 7h, to measure the concentration of PGFM in D15. After treatment administration, uterine biopsies were collected at 1.5h (C1.5h, N=8 and E1.5h; N=10) or 3h (C3h, N=8 and E3h, N=11). The abundance of transcripts for the genes was determined by qPCR. In D14 to D23 the area of the corpus luteum (CL; $cm^2$), blood flow (%) and plasma P4 concentration were assessed daily. Statistical analyzes were performed using the SAS program (version 9.4, SAS Institute Inc., Cary, NC, USA). In D18 to D20, there was a smaller area of CL in Group E (P = 0.023). Group E showed higher concentration of PGFM (P = 0.0002) at 6h (225.45 ± 16.96 pg/mL) and 7h (285.58 ± 33.09 pg/mL) after the application of 17β-E2. In D16 and D17, Group E showed lower concentration of P4 (P = 0.019) in D16 (4.14 ± 0.97 ng/mL) and D17 (2.84 ± 0.79 ng/mL). Functional luteolysis in Group E was anticipated in 1.14 days (17.07 ± 0.43; P = 0.006). Similarly, Group E also showed an anticipation of structural luteolysis in 1.26 days (18.42 ± 0.33; P = 0.026). Among the treatment groups, abundance did not differ for the PKCα (P = 0.79), PRKCβ (P = 0.17), AKR1B1 (P = 0.34) and PTGS2 (P = 0.22) genes. There was a treatment effect only for the transcripts PLA2G4 (P = 0.03) and AKR1C4 (P = 0.05), however, the abundance of both decreased in Group E at 1.5 and 3.0 h after 17β-E2 administration. It is concluded that the application of 17β-E2 at D15 promoted an increase in PGFM concentrations and the anticipation of functional and structural luteolysis in Nellore heifers; however, this increase was not associated with an increase in the gene transcription of the studied proteins. Acknowledgement: FAPESP, CAPES

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Embryology, developmental biology, and physiology of reproduction

# Immunolocation of caspase 3 in corpus luteum of bovine females treated with Dinoprost Tromethamine or Sodium Cloprostenol, with full or half dose, during metestrus or diestrus - previous results

**Ana Clara Degan Mattos[1], Gabriel Artur Marciano do Nascimento[3], Rosemeri de Oliveira Vasconcelos[1], Paulo Henrique Leal Bertolo[1], Carlos Eduardo Bezerra de Moura[2], Vanessa Garcia Rizzi Mussi[3], Lindsay Unno Gimenes[1]**

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The aim of the present study was to evaluate the luteolytic efficiency of the drugs Dinoprost Trometamine (DT) and Sodium Cloprostenol (SC), in full (100%) or half dose (50%), in metestrus or diestrus (D4 or D11 post-ovulation, respectively), regarding apoptosis, by the caspase 3 immunolocation. For this purpose, 40 *Bos indicus* or crossbred cows were submitted to an ovulation synchronization protocol. The time of ovulation was monitored by ultrasound evaluation and only animals which ovulated between 72 and 84 hours were allocated to the experimental groups. A total of 23 cows were selected for containing corpus luteum (CL) and were divided into the following treatments, in a factorial arrangement 2 (day of the estrous cycle) x 2 (luteolytic agent) x 2 (dose): D4CS50%, D4CS100%, D4DT50%, D4DT100%, D11CS50%, D11CS100%, D11DT50% and D11DT100% (n = 3/ group, except D11DT50% in which n = 2). After two days of treatment, the animals were slaughtered, their ovaries collected and the CLs isolated. One slide per animal was made, performing immunohistochemical analysis for apoptosis with primary anti-caspase-3 antibody. In each slide, 5 fields were recorded and the immunolocation of large and small luteal cells was analyzed by a single observer. For statistical analysis, ANOVA followed by Tukey's test (SAS) was used at 5% significance level. There was a triple interaction (day x luteolytic agent x dose) for the immunostaining of small luteal cells (SLC; P = 0.04). The number of immunostained SLCs was similar between treatments CS50%, CS100% and DT50%, regardless of administration on D4 ($7.20^{cd}$ ± 1.06; $7.93^{bcd}$ ± 1.06; 5.67cd ± 1.06, respectively) or on D11 ($10.13^{abc}$ ± 1.06; $8.47^{abcd}$ ± 1.06; $9.73^{abc}$ ± 1.06, respectively). However, for group DT100%, a greater number of immunostained cells was observed on D11 ($13.00^{a}$ ± 1.30) compared to all treatments on D4 (DT100%: $4.93^{d}$ ± 1.06). Additionally, within the same day of the estrous cycle (D4 or D11), the groups had a similar number of SLC between treatments. For large luteal cells (LLC) only a dose effect occurred (P = 0.02); CL of animals treated with 50% of the dose of luteolytic agents showed a greater number of positive LLG for caspase-3 when compared to those treated with 100% of the dose (1.05 ± 0.16 vs. 0.52 ± 0.17, respectively). In conclusion, the luteolytic efficiency was higher in SLC when 100% of the dose of DT was administered on D11 compared to the other treatments on D4, while in LLC when 50% of the dose was administered, regardless of the luteolytic agent and the day of the estrous cycle. Financial Support: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Process: 2019/03690-4

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Embryology, developmental biology, and physiology of reproduction

# Small extracellular vesicles from follicular fluid with different progesterone levels modify miRNAs profile in bovine cumulus cells during in vitro maturation

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Follicular environment is exposed to different progesterone (P4) levels during the estrous cycle. This hormone causes molecular modifications in follicular cells (FCs). In follicular fluid (FF), small extracellular vesicles (EVs) can mediate cell communication among FCs affecting biological processes. Thus, the hypothesis of the present study is that EVs from FF with different P4 levels regulates cumulus cells (CCs) miRNAs profile after supplementation during *in vitro* maturation (IVM). For this purpose, local slaughterhouse ovaries were collected in pairs and separated in groups of high (n=5) and low (n=4) follicular P4 levels, defined based on corpus luteum morphological characteristics, related to the early or middle diestrus, and by the follicular P4 concentration (low P4- 63.62±6.79ng/mL Vs high P4- 158.8±17.47ng/mL) (Ávila et al., *Biology of Reproduction, 102*:362, 2019). The 3-6mm follicles were aspirated to collect cumulus–oocyte complexes (COCs) and EVs. To pellet small EVs, FF was ultracentrifuged twice at 119700xg for 70 minutes. After, COCs (n=20/group) were divided in four groups for supplementation (1:1 volume of EVs) experiments during IVM. The IVM (100µL) medium (TCM199) was divided into 1) 10% complete Fetal Bovine Serum (FBS); 2) 10% EVs free-FBS; 3) 10% EVs free-FBS supplemented with EVs-high-P4 and 4) 10% EVs free-FBS supplemented with EVs-low-P4 groups. After 9 hours of IVM, CCs were collected for total RNA extraction (*miRNeasy Mini Kit* ), mature miRNA reverse transcription using miScript II RT Kit (Hispec buffer); and relative expression analysis of 383 bovine miRNAs by RT-PCR using miScript SYBR Green PCR Kit. The data were analyzed by ANOVA considering significance level of 5%. A total of 233 mature miRNAs were detected in all CCs matured groups. Four miRNAs were differently modulated by FBS EVs presence; three miRNAs upregulated by complete FBS (miR-133a, miR-411a, miR-582) and one upregulated by EVs free-FBS (miR-382). A comparison between EVs-free FBS and EVs-high-P4 demonstrated increased levels of two miRNAs (miR-154c, miR-411a) and one (miR-582) down regulated in CCs treated with EVs-high-P4. MiR-411a was upregulated in CCs treated with EVs-low-P4 when compared with EVs-free FBS. A comparison between EVs-high-P4 and EVs-low-P4 demonstrated two miRNAs highly expressed (miR-154c and miR-582) in EVs-low-P4 supplemented CCs. Bioinformatics analysis (miRWalk 3.0) identified relevant pathways predicted to be modulated by all differentially expressed miRNAs, such as ErbB signaling (22 genes), progesterone-mediated oocyte maturation (18 genes), GnRH signaling (19 genes), and endocytosis (48 genes). In conclusion, FF EVs of different P4 concentrations supplemented during IVM alter the CCs miRNAs profile. Thus, it is suggested that P4, through small EVs can modulate different molecular routes in CCs. Funding: FAPESP 2014 / 22887-0, CNPq 420152 / 2018-0 and CAPES finance code 001.

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Embryology, developmental biology, and physiology of reproduction

## Use of the synthetic peptide EVP50 for activation of bovine oocytes

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Fertilization in mammals is characterized by oscillations in intracellular calcium concentration that are responsible for oocyte activation. The interest in oocyte activation mechanisms has increased due to embryo biotechnologies, such as cloning by nuclear transfer and intracytoplasmic sperm injection. Vipericidins comprise the cathelicidin-related antimicrobial peptides from the venom gland of the pit vipers and previous studies shown that EVP50, a vipericidin encrypted nonapeptide, induced extracellular calcium influx and intracellular calcium release. Therefore, it has been hypothesized that EVP50 may cause oscillations in intracellular calcium concentration in oocytes. Thus, the aim of this study was to evaluate EVP50 for activation of bovine oocytes. EVP50 was obtained through solid phase synthesis and with a purity higher than 95% that was confirmed by high performance liquid chromatography and mass spectrometry. Ovaries were collected in a local abattoir and follicles (2-8 mm) were punctured. The COCs recovered were subjected to IVM for 26 h. Mature oocytes were allocated in five experimental groups: control (ionomycin 5 µM) and EVP50 (0, 1, 10 and 40 µM) incubated for 4 min. After, oocytes were incubated with 6-DMAP (2 µM) during 4 h in SOF medium and then submitted to IVC in SOF medium for seven days. The rates of cleavage and blastocyst production were evaluated on the second and seventh day of culture, respectively. Analysis was performed using Minitab software. Data are presented as percentage (± SD) and compared by Fischer's exact test. Differences were considered significant at the 5% level of significance. In total, 1172 oocytes were obtained from 11 replicates. Oocytes were distributed as follow: ionomycin (250) and EVP50 0 µM (155), 1 µM (214), 10 µM (286) and 40 µM (273). The control group (ionomycin) was always significantly superior ($P<0.05$) to the other groups, both in the rate of cleavage (80.0 ± 15.3%) and blastocyst production (35.6 ± 10.8%). Comparing cleavage rates among EVP50 groups, we observe that 1 (19.6 ± 10.4%) and 10 µM (22.4 ± 12.4%) were higher ($P<0.05$) than 0 µM (8.4 ± 6.3%) and similar ($P>0.05$) to 40 µM (14.7 ± 12.9%). As for the production of blastocysts, the best ($P<0.05$) results were obtained using 40 µM (2.2 ± 3.7%), when compared to 0 (0.6 ± 0.5%), 1 (0.0%) and 10 µM (1.8 ± 3.7%). In conclusion, EVP50 has the potential to activate bovine oocytes, especially in higher concentrations. However, further studies in relation to incubation time and testing of other concentrations are necessary. This study was financed by PRONEX/FUNCAP (grant PR2-0101-00059.01.00-15). Keywords: Embryo, Parthenogenesis, Peptide, Vipericidins.

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Embryology, developmental biology, and physiology of reproduction

# Hyperspectral and confocal imaging of cleavage stage bovine embryos can differentiate between on-time and fast-developing embryos metabolism

**Clara Ana Santos Monteiro[1,2], Darren Jin Xiang Chow[2], Gabriela Ramos Leal[1,2], Tiffany Cheow Yuen Tan[2], Ana Maria Reis Ferreira[1], Jeremy G Thompson[2], Kylie R Dunning[2]**

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Embryo quality can predict implantation potential, being central to a successful IVP program. Metabolic and morphokinetic analyses have immense potential as assessments in this regard and although they are related, surprisingly, few studies to date have linked these factors, especially during early development. Thus, the present study aimed to compare embryos with different developmental kinetics at 48- and 96-hours post-fertilization (hpf), considering the metabolic activity, using optical imaging, and DNA damage. Embryos were fixed at 48 (Day 2; n=40, in three replicates) and 96 (Day 4; n=24, in two replicates) hpf and divided into groups according to their developmental kinetics: on-time (Day 2: 2 cell (n=5); Day 4: 5-7 cell (n=15)) or fast-developing (Day 2: 3-7cell (n=35); Day 4: 8-16 cell (n=9)). Metabolic activity was assessed by cellular autofluorescence: NAD(P)H, FAD, and the optical redox ratio (FAD/(FAD+NAD(P)H)) using laser confocal microscopy and a wide range of endogenous fluorophores by hyperspectral microscopy, a novel, and non-invasive optical imaging approach. DNA damage was determined using γH2AX immunohistochemistry. All data were checked for normality and log10 transformed when required. Intensities of endogenous fluorophores (confocal and hyperspectral microscopy) and γH2AX quantification were analyzed by t-test or Mann Whitney test for data not following a normal distribution. The analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc.) and differences were considered to be significant at $P < 0.05$. At Day 2 and 4, fast-developing embryos showed a significantly lower abundance of endogenous fluorophores in hyperspectral imaging, indicating lower metabolic activity. Interestingly, fast-developing embryos, on Day 2, contained lower levels of DNA damage. At Day 4, fast-developing embryos showed significantly higher levels of NAD(P)H on confocal imaging, leading to a significantly lower optical redox ratio compared to on-time embryos. Collectively, these results demonstrate that fast-developing embryos present a different metabolic pattern on Day 2 and Day 4 of development, compared to on-time embryos. The use of hyperspectral imaging detected 'quiet metabolism' in these embryos. To our knowledge, this is the first collective use of these forms of optical imaging in early cleavage cattle embryos, and our results are promising for its application to embryo selection. This study was funded by the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics (CE140100003) and in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. Keywords: Morphokinetic, Autofluorescence, FAD, NAD(P)H, Redox ratio, DNA damage.

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Embryology, developmental biology, and physiology of reproduction

# "FLI" supplementation during IVM does not alter embryo production after IVF in cattle

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Adequate oocyte maturation optimizing oocyte developmental competence is crucial for assisted reproductive technologies applied to animals and humans. Therefore, in the bovine IVF context, IVM constitutes a fundamental step and opportunity to improve overall outcomes. Previous studies in pigs suggest that the addition of FLI [a combination of fibroblast growth factor-2 (FGF-2), leukemia inhibitory factor (LIF), and insulin growth factor 1(IGF-1)] to the IVM medium improves nuclear maturation and embryo production. However, no previous study has tested this combination in IVM/IVF in cattle. The aim of the present study was to assess the effects of FLI addition to IVM media utilizing FSH in supraphysiological concentrations (traditional approach; FSH) or FSH and steroids at approximately physiological levels associated with amphiregulin [follicular system (FS); Soares *et al.,* Reproduction, Fertility and Development, 29:2217-2224, 2017]. The base medium for all groups consisted of TCM199 (with Earle's salts, bovine serum albumin, pyruvate, and amikacin). The FS medium was supplemented with $10^{-2}$ UI/mL rhFSH, 100 ng/mL amphiregulin, 50 ng/mL 17β-estradiol and 150 ng/mL progesterone, while the conventional medium just supplemented with $10^{-1}$ UI/mL rhFSH. The combination FLI was composed of 40 ng/mL FGF2, 20 ng/mL LIF and 10 ng/mL IGF-1. Five replicates were performed to compare four experimental groups: FSH; FSH+FLI; FS; FS+FLI. Ovaries were obtained from a slaughterhouse and COCs recovered by aspiration were submitted to IVM for 24h, followed by *in vitro* fertilization for 18h. Presumptive zygotes were culture for seven days at 38.5°C under 5% $CO_2$, 5% $O_2$ and 90% $N_2$ in humidified air. Blastocyst rate was calculated in relation to total oocytes and blastocyst cell numbers were assessed after Hoechst 33342 staining. Rate of expanded/hatched blastocysts was calculated in relation to total blastocysts. Data were arcsine transformed and compared with Tukey (parametric data) or Wilcoxon (non-parametric data) tests. The addition of FLI during IVM did not affect blastocyst rate (P>0.05; FSH 30.28±3.65; FSH+FLI 34.91±5.15; FS 34.73±1.50; FS+FLI 34.36±3.21), expanded/hatched blastocyst rate (P>0.05; FSH 90,39±2.64; FSH+FLI 86,04±5.03; FS 92,87±3.38; FS+FLI 85,97±5.82), nor total embryo cell number (P>0.05; FSH 198.02±11.81; FSH+FLI 180.50±9.52; SF 194.05±11.44; SF+FLI 184.83±13.46) in any of the base media tested. In summary, these results suggest that FLI addition to the IVM medium does not positively impact on bovine IVP. Further studies are needed to assess the impact of FLI on pregnancy and birth rates following IVF. Supported by FAPESP 2019/14588-6.

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Embryology, developmental biology, and physiology of reproduction

# Use of different methods to assess abundance of genes stimulated by the conceptus 20 days after IATF in dairy cattle

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We aimed: 1) to evaluate the abundance of two genes stimulated by the conceptus using samples collected by four methods (peripheral blood mononuclear cells [PBMC], whole blood, cervical cytology or immune cells from milk); and 2) to compare these four methods as pregnancy predictors on day 20 after timed-AI (TAI) in dairy cattle. Eighteen Holstein females (12 cows and 6 heifers) with BCS of 3.1±0.3 (1 to 5 scale), were submitted to an E2/P4-based protocol to synchronize ovulation for TAI (D0). On D20 post-TAI, blood samples were collected from coccygeal vessels for isolation of PBMC and in Tempus Blood RNA® tubes. Isolation of PBMC was performed by Ficoll® Paque Plus gradient. Samples of cervical cytology were collected using a cytological brush. Milk samples were collected before routine milking in cows, and immune cells were isolated as proposed by Schanzenbach et al. (Plos One, 12: 2, 2017). The RNA from PBMC, cervical cytology, and milk were extracted using Trizol® Reagent according to manufacturer's instructions. Pregnancy diagnosis was performed on D30 using transrectal ultrasonography and females were classified as pregnant (P; n=8) or non-pregnant (NP; n=10). Expression of target genes (*ISG15* and *LGALS3BP*) was quantified by RT-qPCR and normalized in relation to the reference genes (*GAPDH* and *PPIA* for PBMCs; and *GAPDH* and *ACTB* for whole blood, cervical cytology and milk). Data were analyzed by ANOVA using the PROC MIXED procedure (SAS). Abundance of *ISG15* was greater in the P group than in NP group for PBMC (0.08 ± 0.01 vs. 0.03 ± 0.01; P=0.004), whole blood (0.024 ± 0.003 vs. 0.014 ± 0.004; P=0.04) and cervical cytology (0.41 ± 0.12 vs. 0.13 ± 0.08; P= 0.04). No difference (P>0.58) was detected for milk samples between the P and NP groups. For *LGALS3BP* abundance, no difference was detected between P and NP groups for PBMC (P=0.31), whole blood (P=0.43), and milk (P=0.65), but a tendency for greater abundance in P group was observed for cervical cytology (0.035 ± 0.006 vs. 0,021 ± 0.004, P=0.07). When the fold change between the *ISG15* abundance in P and the mean of NP animals was compared among the four methods, a greater (P<0.001) fold change was observed in cervical cytology (3.22 ± 1.54) than in the PBMC (2.75 ± 0.26), whole blood (1.71 ± 0.25) and milk (0.005 ± 0.002). ROC curve analysis indicated that *ISG15* abundance was a significant (P<0.001) predictor of pregnancy in PBMC (AUC= 0.92), but not in whole blood (AUC=0.68, P=0.16), cervical cytology (AUC = 0.71, P=0.42) or milk (AUC=0.42, P=0.64) methods. In conclusion: I) milk method is not a good indicator of genes stimulated by pregnancy; II) although an increased *ISG15* abundance is observed in P dairy females for whole blood and cervical cytology, the PBMC method is the best pregnancy predictor on D20 post-TAI; and III) the use of *LGALS3BP* abundance for determination of pregnancy status is not indicated for any method. Acknowledgments: FAPESP (2015/10606-9; 2019/16040-8).

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# In vivo and in vitro-produced bovine blastocysts secrete small extracellular vesicles with different miRNAs content

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*In vivo* and *in vitro*-produced bovine embryos present different metabolic profiles, gene transcription, and distinct ability to establish and maintain the pregnancy. Pregnancy losses may occur due to communication failures between embryo and mother. Small extracellular vesicles (sEVs) are part of the embryo-maternal crosstalk and carry bioactive molecules, such as microRNAs (miRNAs). These are small non-coding RNA molecules involved in post-transcriptional regulation and may play a role in modulating embryo-maternal communication during early pregnancy. Our hypothesis is that sEVs secreted by *in vivo* and *in vitro*-produced bovine embryos have different miRNA profiles. Nellore cows previously synchronized were super-stimulated with FSH to produce *in vivo* and *in vitro* embryos. On day 7 after fertilization, embryos from both groups were individually cultured for 48 hours in 30 µL of modified SOFaaci to obtain the conditioned medium (CM). Only CM in the presence of hatched embryos were used. Four pools of CM from 8 embryos each (240 µL of CM each pool) per group were used to isolate sEVs using Exoquick-TC (1:1). sEVs pellets were used for total RNA extraction. MiRNA reverse transcription was performed using miScript II RT Kit (HiFlex). Relative abundance of 382 bovine miRNAs were determined by RT-PCR data normalized by the geometric mean of miR-99b and Hm/Ms/Rt U1 snRNA. Differences in relative abundance were determined by Student's t-test. A total of 106 miRNAs were identified in both groups of sEVs. In sEVs from *in vivo* embryos, 14 miRNAs were upregulated, while two miRNAs were increased in sEVs from *in vitro* embryos. Enriched pathways modulated by these miRNAs were determined by bioinformatics analysis using mirWalk software (version 3.0). The miRNAs (miR-92b, miR-296-5p, miR-323, miR-382, miR-421, miR-541, miR-669, miR-935, miR-940, miR-1225-3p, miR-1249, miR-1281, miR-1296 and miR-1343-3p) were increased in sEVs from *in vivo* embryos and are predicted to regulate MAPK (158 genes), Ras (148), chemokine (101), oxytocin (89) and cell adhesion molecules (CAMs) (87) pathways. Furthermore, miR-494 and miR-1246, which were upregulated in sEVs from *in vitro* embryos are predicted to modulate Wnt (10), CAMs (9), hypoxia-inducible factor 1 (HIF-1) (7) and lysine degradation (5) signaling pathways. These results demonstrate that embryos produced under different conditions (*in vivo* vs. *in vitro*) secrete sEVs with different miRNA profiles. Moreover, miRNAs carried by *in vivo* embryonic derived sEVs are predicted to regulate important endometrial pathways, like oxytocin, MAPK (ERK1⁄2) and Ras, while miRNAs present in sEVs secreted by *in vitro* derived embryos can be involved in regulation of lysine degradation. Based on these findings we suggest that bovine embryo sEVs could modify embryo-maternal crosstalk during early pregnancy and consequently affect pregnancy establishment. Funding: FAPESP 2017/19681-9, 2014/22887-0 and 2018/13155-6. Acknowledgments: CRV Lagoa.

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ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Expression of RNAm for galectin 3 binding protein in the circulating immune cells and vaginal mucosa cells during early pregnancy in beef heifers

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Galectin 3 binding protein (*LGALS3BP*) is a molecule stimulated during early pregnancy and has a crucial role in the process of cell adhesion in the bovine endometrium. We aimed: 1) to evaluate the expression of *LGALS3BP* transcript using vaginal cytology as an alternative to indicate pregnancy status; and 2) to compare this method with the isolation of peripheral polimorphonuclear blood cells (PMN) to determine *LGALS3BP* expression on day 20 after timed-AI (TAI) in beef heifers. Nelore heifers (n=31) weighting 422 ± 47.8kg were submitted to an E2/P4 based protocol to synchronize ovulation for TAI (D0). On days 16,18 and 20, samples of vaginal cells were collected using cytological brush (Cytobrush; Viamed Ltd, West Yorkshire, UK), which was placed in the fornix surrounding the external cervix os to recover the superficial cells from vagina, in which the samples were stored in liquid nitrogen until RNA extraction. On D20, blood samples collected from jugular vein (30ml) were used for PMN isolation (Zymo Research, CA, USA). The presence of an embryo with heartbeat on D30 indicated if the animals were Pregnant (Preg n=16) or Non-Pregnant (NPreg n=15). Extracted RNA was treated with DNase I for genomic DNA contamination before synthesis of complementary DNA (cDNA) using 1000ng (Cyto) and 500ng (PMN) of total RNA. The *LGALS3BP* expression was evaluated by RT-qPCR in the vaginal cell samples on D16, 18 and 20 (n=6 Preg and 7 NPreg), and on D20, the relative *LGALS3BP* expression between Preg and NPreg heifers was compared between the vaginal cells and PMN (n=14 Preg and 13 NPreg/cell type). Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) and Actin Beta (*ACTB*) were used for normalization of relative expression in both cell types. For the comparison between the methods, the relative *LGALS3BP* expression on D20 in each Preg heifer was divided by the averaged expression in the NPreg. The results were analyzed by ANOVA and PROC MIXED procedure (SAS), considering the main effects of group (G), time (T) and its interaction (TG). No significant effects (P>0.75) of G, T and TG were observed for the *LGALS3BP* expression in vaginal cells from D16 to 20. Also, when evaluated on D20 with a large number of animals, no significant difference was observed in *LGALS3BP* expression between Preg and NPreg for the vaginal cells (relative expression to reference genes: 0.018±0.007 vs 0.004±0.001 P=0.29) and PMN (0.013±0.002 vs 0.01±0.003 P=0.46). When compared the relative *ISG15* expression in the Preg to NPreg group in the two cell types, no difference (P=0.43) was found between the PMN (fold change 1.22) and vaginal cells (fold change 4.23). The coefficient of variation within each group in samples analyzed on D20 ranged from 77% to 131% for vaginal cells, and 53% to 73% for PMN. In conclusion, the high variation in *LGALS3BP* expression within Preg and NPreg heifers impairs its use as an effective pregnancy predictor in cattle. Acknowledgmen FAPESP (Grant number: 2019/05926-5).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# High body energy reserve cows have different mirnas profile in isthmus oviductal cells during early embryonic development

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Body condition score (BCS) can influence reproductive performance by changes in the follicular and oviduct environment, impacting reproductive efficiency by modulation of important physiological pathways within the oviduct affecting early embryonic development. Thus we tested the hypothesis that cows with high or moderated body energy reserves have different miRNAs profile in isthmus oviductal cells. For this, a homogeneous group of dry Nelore cows (495,67± 39,66 Kg and BCS=5,5 ± 0,48, in a 1 to 9 scale) were fed in a calan gate system for ~100 days and randomly assigned to 1 of the 2 feeding treatments: 1) cows receiving an *Ad libitum* diet to increase body energy reserves (HBER) and 2) cows fed 70% of the HBER group ingestion to mantain body weight and BCS (MBER). Animals were submitted to fixed-time artificial insemination and ~120 hours after ovulation induction, cows were slaughtered. Next, the ipsilateral oviduct to the corpus luteum was collected and dissected. Isthmus luminal epithelial cells were obtained only from animals whose embryos were recovered at the 8-cell stage (HBER n=3; MBER n=3). At the end of the feedlot period, body weight was 642,33 ± 50,41; 482 ± 38,76 Kg and BCS 6 ± 0,57 , 4,66 ± 0,33 in HBER and MBER, respectively. Additionally, animals presented similar serum biochemical profile. Mature miRNAs were reverse transcribed using miScript HiSpec Buffer and the profile of 383 bovine mature miRNAs was analyzed by qPCR in isthmus cells obtained from both groups. The relative levels were evaluated and data normalized by the geometric mean of miR-99b and Hm/Ms/Rt U1 snRNA. To identify differential miRNA expression between groups, Student's t-test was performed ($p<0,05$). A total of 243 miRNAs were identified in both groups; out of these, six miRNAs (miR-148a, miR- 192, miR-28, miR-296-5p, miR-664b and miR-1271) were upregulated in HBER group, and two miRNAs (miR-190a and miR-378d) were increased in MBER group. Bioinformatics analysis using miRWALK 3.0 platform demonstrated that the upregulated miRNAs in HBER group are predicted to modulate signaling pathways such as MAPK (77 genes), RAS (68), Hippo (45), Insulin (42), and Insulin Resistence (30). Moreover, the increased miRNAs in MBER group are predicted to regulate RAS (37 genes), WNT (25), mTOR (22), FoxO (19), and Insulin (19) pathways. These results show that high BER conditions modify the isthmus cell miRNAs profile when compared with moderate BER cows. Furthermore, pathways related to cell proliferation, differentiation and metabolism, such as Insulin signaling, are predicted to be regulated by miRNAs in both groups. However, the Insulin Resistence pathway is predicted to be regulated only by the HBER group. Thus, we suggest that high BER can metabolically modulate the oviduct tissue and possibly modulate important signaling pathways affecting early embryonic development and reproductive efficiency in cows. Funding CAPES (001), FAPESP (2014/22887-0), CNPq (#420152/2018-0).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Effect of the calving season (rainy x dry) on uterine inflammatory response and energy reserve in beef cows

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The aim of this study was to evaluate the uterine health and energy status of beef cows calved in the rainy and dry season in the amazon biome. This experiment was performed between August and November 2019 at Embrapa Rondônia experimental farm. Nelore cows (*Bos indicus*, n = 19), 3 years old, were assessed at -20, -10 days prepartum and 7, 14, 21, 28, 35, e 42 days postpartum (DPP). In the experimental period, the animals were weighed, and internal angle of the rump (IAR) and subcutaneous fat thickness (SFT) were measured Blood samples were collected to measure the serum concentration of β-hydroxybutyrate (BHB) and glucose (Ketovet®, Brazil; Accu-Chek® Active, Roche, Brazil; respectively). On days 7, 14, 21, 28, 35, and 42 postpartum, the cows were subjected to evaluation of uterine discharge using a vaginoscope and cytological collection of uterine tissue using the cytobrush technique (Cardoso et al., Reproduction in Domestic Animals 52: 1153-1157, 2017). Uterine discharge was graded on a scale from 0 to 3 (0 = mucus, 1 = mucus with flecks of pus, 2 = ≥50% purulent exudate, 3 = hemorrhagic and / or purulent exudate), as adapted from Williams et al. Theriogenology: 63, 102-117, 2005. The samples collected from uterine tissue were fixed on glass slides, stained with a Panotic kit (RenyLab®, Barbacena, Brazil) and subjected to 200 cell count in an optical microscopy. The cows were divided into 2 experimental groups according to the period of calving: 1) cows calved during the dry season (August) (n = 12), and 2) cows calved in the rainy season (October and November) (n = 7). The variables were analyzed using repeated measures (PROC MIXED, SAS®, 1998). Climatological data were obtained in a local monitoring station. The average temperatures were 26,3 °C in the dry season and 25,9,6 °C in the rainy season. The average humidity was 62,5% in the dry season and 82,3% in the rainy season. The precipitation averages were 1,1 mm/day and 10,1 mm/day in the dry and rainy periods, respectively. Cows calved in the dry season had a higher ($P<0,05$) concentration of glucose (64,0 ± 1,6 and 56,2 ± 1,8 mg/dL in dry and rainy season, respectively) and BHB (0,93 ± 0,1 and 0,86 ± 0,1 mmol/dL in dry and rainy, respectively), SFT (4,3 ± 0,3 and 4,0 ± 0,2 mm in dry and rainy season, respectively) and uterine discharge (1,4 ± 0,2 and 0,9 ± 0,4 in dry and rainy season, respectively). In contrast, cows calved in the rainy season had higher weight (511,7 ± 8,8 and 531,9 ± 6,8 kg in dry and rainy season, respectively; $P<0,05$). No effect of season ($P>0,05$) was observed in the IAR (110,6 ± 1,3 and 112,6 ± 0,5 º in dry and rainy season, respectively) and proportion of PMN cells (18,3 ± 4,0 and 14,3 ± 3,3 % in dry and rainy season, respectively). The results of this study demonstrate that the calving season has an influence on the energy reserve and uterine health of Nellore cows. Keywords: bovine, beef, postpartum, season.Acknowledgments: FAPERO, EMBRAPA, CNPq and CAPES.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Changes in the antral follicle count and ovarian characteristics from prepubertal to pubertal period in heifers

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The beginning of the reproductive life of Nelore heifers is the focus of study of many groups due to its importance for the animal protein production system. Some of these studies associate individual characteristics of the reproductive system with fertility. The objective of this study was to evaluate ovarian characteristics and antral follicle count (AFC) in heifers from prepubertal to pubertal period. Nelore prepubertal heifers (n=20), 16 months of age, 272,3 ± 23 kg were examined by ultrasound for determining AFC and the area of the ovary. Antral follicle count per $cm^2$ of ovary (AFC density) was also calculated. Additional ovarian ultrasound examinations were performed every 42 days until the detection of a corpus luteum. Once the onset of puberty was determined, all heifers underwent a final ultrasound examination for AFC determination and ovarian measurements. Data from AFC and ovarian measurements were evaluated using paired T test. The average area of the ovary of the prepubertal heifers was smaller than that of the pubertal heifers (5.6 ± 0.2 *vs.* 7.0 ± 0.3 $cm^2$; $P < 0.01$). In contrast, no effect ($P = 0.56$) of the pubertal status on the AFC was detected between prepubertal and pubertal period (19.3 ± 8.0 and 20.3 ± 10 antral follicles, respectively). Numerical difference ($P = 0.07$) was detected for density (AFC/$cm^2$) between prepubertal and pubertal period (3.35 ± 0.23 and 2.82 ± 0.26, respectively). These results demonstrate that the AFC evaluated in the prepubertal period can be used to estimate the AFC in the pubertal period. However, more studies are necessary to elucidate if the AFC maintain constantly during the further stages of the heifer development. Keywords: Ovary. Follicles. Puberty. Fertility. Nelore. Acknowledgements: This research project was supported by CNPq (Project 407307/2016-8).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Effects of estradiol on PGF2α synthesis in beef heifers submitted to resynchronization at 14 days after TAI

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We aim to evaluate the effects of estradiol benzoate (EB) or 17β-estradiol (E2) associated to P4 in the PGF2α synthesis 14 days post-TAI in beef heifers. Nelore heifers were submitted to timed-AI (TAI; D0). On D14, heifers received an intravaginal P4 device (1g, Sincrogest, Ourofino Saúde Animal) and were randomly split: Control (C; no treatment; n=12); EB (1mg EB, Sincrodiol, Ourofino; n=10); or E2 (1mg E2 + 9mg P4, Betaproginn, Boehringer-Ingelheim; n=10). On D14, blood samples were collected hourly for 8 hours (H) after the treatments (H0 to 8) to measure plasma concentrations of PGF2α metabolite (PGFM; pg/mL) by an in-house ELISA. Devices were removed on D22 and pregnancy was diagnosed on D28. Data were analyzed by ANOVA (PROC MIXED), LSD test or Fisher's exact test of SAS. Pregnancy was detected in 17 heifers: C (n=7), EB (n=5) and E2 (n=5) groups. Regardless of pregnancy status, a group by hour interaction ($P<0.05$) indicated increased PGFM concentrations between H4 and 6, and 7 and 8 for E2 and EB groups, respectively; whereas no difference was observed in C group. PGFM concentrations were greater ($P<0.05$) in E2 and EB groups than in C group, respectively, at H5 to 8 and at H8. A greater ($P<0.05$) number of heifers with a CV-identified prominent PGFM pulse (>100pg/mL) was observed in E2 group (60% [6/10]) than in EB (10% [1/10]) and C (0% [0/12]) groups. The peak (pg/mL) and area under pulse curve (AUC; pg/mL/h) of PGFM pulses were greater ($P<0.05$) in E2 group (118 ± 20 and 162 ± 36) than in C group (31 ± 4 and 30 ± 4), but did not differ ($P>0.1$) from EB group (72 ± 30 and 90 ± 47). Amplitude (pg/mL) of PGFM pulse was greater ($P<0.05$) in E2 group (91 ± 19) than in EB and C groups (overall mean: 27 ± 15). Maximum PGFM concentration (pg/mL) did not differ ($P>0.1$) between E2 (120 ± 20) and EB (80 ± 18) groups and both were greater ($P<0.05$) than C (37 ± 4). For non-pregnant heifers, a group by hour interaction indicated increased ($P<0.05$) PGFM concentrations in E2 group from H4 to 8, and in EB group at H7 and 8. PGFM pulse concentrations at the peak, amplitude and AUC were greater ($P<0.05$) in E2 group (124 ± 23, 93 ± 24 and 151 ± 48) than in C group (26 ± 3, 10 ± 1 and 23 ± 2); whereas the EB group (88 ± 43, 61 ± 39 and 115 ± 70) did not differ ($P>0.1$) from the other groups. Maximum PGFM concentration did not differ ($P>0.1$) between E2 (124 ± 23) and EB (110 ± 30), but was greater ($P<0.05$) in both groups than in C (32 ± 3). For pregnant heifers, no effects of group, hour or their interaction were detected, but maximum PGFM concentration was greater ($P<0.05$) in E2 (115 ± 34) than in EB (49 ± 10) and C (41 ± 5) groups. Prominent PGFM pulses were only detected in pregnant heifers from E2 group (3/5). In conclusion, PGF2α synthesis is more stimulated and anticipated in heifers treated with E2 than EB; and 1mg of EB 14 days after TAI does not induce PGF2α synthesis in pregnant beef heifers. Acknowledgments: FAPESP (2015/10606-9; 2017/18613-0).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Expression of prostaglandin receptor in bovine pituitary gonadotrope cells

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Prostaglandin F2α (PGF) has been associated to ovulation in cattle (Pfeifer et al, Pesquisa Agropecuária Brasileira, 51:738-734, 2016), but its mechanism of action is unclear. Based on this, we hypothesized that there are PGF receptors in the pituitary gland, and that PGF receptors are colocalized with gonadotropes. The aim of this study was to evaluate the presence of PGF receptors in pituitary luteotropic cells. Cow pituitaries (n=3) obtained from a local slaughterhouse were used for this study. Soon after collection, pituitaries were fixed during 24h in formaldehyde. Pituitaries were embedded in paraffin, cut into 20µm sections and mounted on slides using standard procedures. For the immunohistochemistry, slides containing pituitary sections were submerged in xylene (10 min) and after in 100%, 95% and 70% ethanol (10, 5 and 5 min, respectively). Sections were washed in distilled water and PBS for 5 min. Slides were then submerged in sodium citrate buffer 0.01M pH 6.0 in the microwave for about 10 minutes. After that, the slides were submerged in PBS plus BSA 1%, as a blocking agent, for 45 min. After blocking, sections were incubated (overnight, 4ºC) in a mix containing the two primary antibodies; lutropin β (C-6) monoclonal antibody (Santa Cruz Biothecnology, Inc.) at 1:100 dilution, to identify LH cells, and FP receptor polyclonal antibody (Cayman Chemical Company) at 1:100 dilution to identify PGF receptors. After 24 h, the sections were washed in PBS again and incubated in a dark room with Donkey anti-mouse Alexa Fluor®488 (green, Abcam®) secondary antibody to stain LH cells and ST Alexa 546 (red, Abcam®) at 1:2000 dilution for staining PGF receptors. Furthermore, cell nuclei were counterstained with DAPI. To analyze the immunofluorescent colocalization of PGF and LH receptors, the bright-field images were captured using an Olympus (Center Valley, PA) Provis microscope fitted with a Kodak (Rochester, NY) DCS 330 digital camera. Colocalization of PGF receptor and LH was examined by merge across each slide. As result, no colocalization between gonadotropes and PGF receptors was detected in any of pituitary samples analyzed. This result indicate that PGF does not stimulates LH release directly in the gonadotropes.In conclusion, we did not find colocalization between PGF receptors and luteotropic cells in bovine pituitary gland. However, further studies are needed to understand its mechanism of action.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Seasonal variation of apoptotic index in cumulus cells and maturation competence of bovine oocytes

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During summer beef cows grazing native pastures in temperate regions can be exposed to acute heat stress, which may affect the quality of the cumulus oocytes complexes (COCs) compared to colder seasons. The aim of this study was to determine the effect of seasonality on the quality of cumulus cells (CCs) and in vitro maturation (IVM) competence of bovine COCs.Ovaries were collected monthly from slaughterhouse during winter (June to August) and summer (December to February). Apoptotic index was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in CCs from intact immature and in vitro matured COCs from each season. After IVM, meiotic progression of oocytes from each group was assessed by orcein staining. Data on apoptotic index (n=25-35 COCs/group) and the rate of meiotic progression of oocytes was recorded at 24 h (n=57-63 oocytes/group) and analyzed by one-way ANOVA followed by Tukey test. Apoptotic index in CCs of immature oocytes recovered in summer was almost fourfold greater compared to those recovered in winter (1.28 ± 0.08 vs. 0.36 ± 0.01; $P < 0.05$). The percentage of metaphase II matured oocytes tended to be greater for those recovered in winter than in summer (77.77 ± 1.78 vs. 69.09 ± 0.27; $P = 0.06$). In addition, the proportion of TUNEL-positive in CCs of IVM oocytes from summer oocytes was higher than those collected in winter (5.8 ± 0.27 vs 1.76 ± 0.09; $P < 0.05$). In conclusion, higher numbers of CCs with DNA fragmentation during the summer may explain the reduction in oocyte maturation capacity, considering the relevance of the cumulus-oocyte communication during this stage of development.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Metabolic effects of fetal bovine serum removal on in vitro culture of bovine embryos

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Supplementation with fetal bovine serum (FBS) renders embryo culture media as undefined. This can mask results of scientific research, specifically, studies of cell differentiation that are influenced by glucose metabolism, as well as by growth factors present in FBS. In this study, we tested the hypothesis that the removal of FBS during in vitro culture (IVC) does not reduce development rates, but alters aspects related to energy metabolism. Grade I oocytes were collected from commercial slaughterhouse ovaries and subsequently in vitro matured and fertilized. Zygotes were then placed in KSOM medium (Millipore) in an atmosphere of 5% CO2 and 5% O2 and randomly distributed in the following groups: group SFB, in which embryos were supplemented with 5 % (v / v) of SFB (Thermo Fisher) at the fourth day (D4) of IVC, Group KSOM- 30, in which 30% of the medium volume was removed and renewed at D4, and Group KSOM-zero, in which there was no supplementation with FBS and no renewal of the culture medium. In D9, rates of blastocyst formation (blastocysts/total zygotes) and development (blastocysts/cleaved) were recorded. Furthermore, blastocysts were stained with 2.5 mM CellROX (ThermoFisher) and 1 mM MitoTracker Red (ThermoFisher) for 30 minutes or subjected to measurement of NADH and FAD+ upon excitation with 360nm and 488nm, respectively, under an epifluorescence microscope. Fluorescence intensity was measured using Image J (NIH) *software*. Data were analyzed by ANOVA, considering replicate as a random variable, and the comparison of means was performed using Tukey's test in SAS 9.4 software. Results showed that the blastocyst rate (n = 8 replicates) in SFB group (33.61 ± 2.85%) was higher (p = 0.02) than in KSOM-zero group (21.57 ± 2.85%), which tended to be smaller (p = 0.06) than KSOM-30 group (31.48 ± 2.85%). Development rates (n = 8 replicates) tended to be higher in SFB (40.79 ± 3.94%, p = 0.07) and KSOM-30 (39.93 ± 3.94%, p = 0.09) groups than in KSOM-zero group (27.45% ± 2.85%). There were no significant differences in fluorescence intensities of CellROX and MitoTracker (n = 8 embryos per group) or NADH and FAD+ (n = 8 embryos per group). Thus, it was concluded that the removal of FBS reduced blastocyst rates, although this was not observed when there was 30% renewal of the medium in D4. It was also concluded that removal of SFB did not alter the observed metabolic variables. Studies are being carried out to verify the impact of SFB on cell differentiation of IVP bovine embryos. Financial support by FAPESP grants 2017 / 09576-3, 2017 / 25574-0, 2019 / 03014-9.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# The bovine embryo modify the miRNA profile within the ovidutal environment at early diestrus

**Rosane Mazzarella[1], Natália Marins Bastos[1], Gabriella Mamede Andrade[1], Alessandra Bridi[1], Jorge Pinzon[2], Guilherme Pugliesi[3], Luciano Andrade Silva[1], Felipe Perecin[1], Flávio Vieira Meirelles[1], Juliano Coelho da Silveira[1]**

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The oviduct provides the environment for early embryonic development. Its lumen is composed mainly by ciliated and secretory cells, which secrets the oviductal fluid (OF). Among the components of the OF, small extracellular vesicles (sEV) can play a role in cellular communication and through miRNAs modulate important metabolic pathways in oviduct cells and embryos. Recently, it has been shown that the embryo can modify the microenvironment in the female tract to its own favor. Herein, we determined the miRNA content of oviduct epithelial cells (OECs) and of sEV from oviducts exposed or not to the embryo. For this, Nelore cows were synchronized by hormonal protocol and divided in two groups, normal artificial insemination (pregnant) and semen diluent (cyclic). Cows were slaughtered 120 hours after ovulation induction. The isthmus portion of the oviduct ipsilateral to the corpus luteum was dissected and flushed for OF and epithelial cells collection. The embryo presence in the OF was confirmed using a microscope. The sEV were isolated from OF by serial centrifugations and by ultracentrifugation. The profile of 383 miRNAs was evaluated in sEV and OECs from pregnant (n=6) and cyclic (n=6) cows. Total RNA was extracted according to Trizol protocol (Thermo Fisher), reverse transcription was performed with HiFlex Buffer using miSCRIPT II RT kit and RTq-PCR with SYBR Green PCR kit (QIAGEN). Real time PCR data were normalized by the geometric mean of Hm/Ms/Rt U1 snRNA and bta-miR-99b. Statistical analysis was performed by Wilcoxon test considering $p<0.05$ for statistical difference. Comparison between miRNA contents of sEV and OECs within the same group demonstrated a total of 358 miRNAs in sEV and OECs of the pregnant group. Among these, 200 miRNAs were detected in both samples, 52 miRNAs were up and 50 down regulated in sEV. Similarly, in sEV and OECs in the cyclic group 364 miRNAs were detected, among them 239 miRNAs in common. Of these, 37 miRNAs were up and 86 were down regulated in sEV. Functional enrichment analysis performed with miRWalk software (version 3.0) demonstrated that miRNAs differently expressed in sEV compared with OECs in both groups are predicted to regulate pathways such as endocytosis, Ras and MAPK signaling. However, cytoskeleton regulation and focal adhesion pathways were predicted to be strongly modulated by miRNAs down expressed in OECs from the cyclic group, but by up expressed miRNAs in OECs from pregnant cows. These pathways are crucial for intercellular communication, signal transduction, proliferation and survival and could be strongly modulated in OECs with the embryo presence. Thus, we suggest that normal expression of these pathways could favor the early embryonic development and transport during their passage through the oviduct. In conclusion, the embryo presence can modulate the ovidutal environment, more specific the miRNA profile within sEV and OECs. Funding: FAPESP 2014/22887-0, 2019/04981-2 and CNPq 420152/2018-0.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Extracellular vesicles supplementation during oocyte in vitro maturation have the potential to modulate gene expression in produced blastocysts

**Luca Angi Souza, Ana Clara Faquineli Cavalcante Mendes de Ávila, Natália Marins Bastos, Rosane Mazzarella, Alessandra Bridi, Paola Maria da Silva Rosa, Flávio Vieira Meirelles, Felipe Perecin, Juliano Coelho da Silveira**

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*In vitro* embryo production (IVP) rates are generally lower than those obtained *in vivo*. One reason is that the *in vitro* system is unable to reproduce satisfactorily the female reproductive environment and to maintain the oocyte quality that will originate blastocysts (BLs). During follicular and oocyte development, small extracellular vesicles (EVs) carry bioactive molecules that contribute to intercellular communication. These EVs are in the follicular fluid (FF) and their contents can alter according to estrous cycle stages. Therefore, these EVs can be associated with oocyte maturation in a follicular stage-specific manner. Thus, the present study investigated the hypothesis that supplementation during oocyte *in vitro* maturation (IVM) with FF EVs from different stages of the estrous cycle generates different gene expression profiles in produced BLs. For that, FF (3-6mm) samples were collected from slaughterhouse ovaries, grouped by corpus luteum morphological characteristics according to development stage (stage I: day 1 to 4; or stage III: day 11 to 17 of the estrous cycle) and the follicular P4 concentration (stage I:- 63.62±6.79ng/mL or stage III: P4- 158.8±17.47ng/mL; Ávila et al., *Biology of Reproduction, 102*:362, 2019). To isolate small EVs, FF passed through serial centrifugations: 10min at 300xg, 10min at 2000xg, 30min at 16500xg and twice at 119700xg for 70min at 4°C. The cumulus-oocyte complexes (COCs) were obtained from follicles (3-6mm) at random moments in the estrous cycle, and supplemented (1:1 volume) during IVM (100µL) with EVs from stage I (EVs I) or stage III (EVs III) follicles. After IVM (22-24 h), COCs were submitted to IVP using semen from the same bull. BLs produced after seven days in culture (n~14/group) were collected for total RNA extraction, cDNA synthesis and RT-PCR analysis. *OCT4*, *HSPA4* and *BAX* transcripts were analyzed in 3 replicates/group and normalized by the geometric mean of the *PPIA* and *RPL15*. The data were analyzed by the student's t-test ($p < 0.05$). The results demonstrated that there was no statistical difference in the transcripts levels analyzed between groups. However, *OCT4* transcripts showed strong tendency to have higher expression ($p=0.07$) in the EVs I group (0.64 ± 0.05) when compared to EVs III (0.42 ± 0.08). Thus, it is possible that supplementation with EVs during oocyte IVM changes the relative expression of genes in blastocyst produced *in vitro*. These results suggest that EVs from follicles at different stages of estrous cycle have the ability to modulate COCs at molecular levels. However, more studies are needed to validate our hypothesis since the sample size and the statistical analysis demonstrated the need for new repetitions. In conclusion, although IVM corresponds to a process lasting only 24 hours, we believe that EVs can impact the initial embryonic development throughout oocyte improvement during *in vitro* maturation. Funding: FAPESP 2014/22887-0; 2019/21028-7 and CNPq 420152/2018-0.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Use of CRISPR/Cas9 for deletion of the LATS2 gene in bovine embryos - preliminary results

**Caroline Pereira da Costa[1], Marcelo Demarchi Goissis[1], Felipe Eduardo Luedke[1], Mayra Elena Ortiz D´Avila Assumpção[1], Bin Gu[2], Janet Rossant[2]**

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The first cellular differentiation event in mammals consists in the separation between the internal cellular mass (ICM) and the trophectoderm (TE). It is known in mice that the HIPPO signaling pathway, via LATS2 protein kinase, plays an important role in controlling the expression of genes that define the segregation of ICM and TE. In bovine, it is suggested that the process of differentiation may be regulated differently, but HIPPO pathway may still be involved in this biological event. In order to test the hypothesis that LATS2 activity is necessary for differentiation of ICM and TE in bovine embryos, we performed microinjections in bovine zygotes to induce genetic deletion of LATS2, using CRISPR/Cas9 system. CRISPR/Cas9 system design was performed in silico using the specific sequence of the LATS2 gene (Gen Bank accession number XM_025000092.1). Two RNA guide sequences (gRNA) were then designed using CRISPR RGEN Tools online software. The forward and reverse oligonucleotide sequences were commercially synthesized, then in vitro annealed, phosphorylated, and cloned in pX330-U6-Chimeric_BBh-hSpCas9-mSA (modified from Addgene) plasmid. DNA sequences corresponding to each gRNA (LATS2 gRNA) were amplified from the generated vector and in vitro gRNA synthesis was performed using the MEGAshortscript™ T7 Transcription Kit (Thermo Fisher). Bovine oocytes were then aspirated from commercial slaughterhouse ovaries, in vitro matured, fertilized and randomly distributed among three groups: Control group without microinjection, Cas9 group, in which zygotes were microinjected with only 70ng/ml of Cas9 enzyme (Sigma), and LATS2 group, in which zygotes were microinjected with 12.5ng/ml of each LATS2 gRNA and 70ng/ml of Cas9 enzyme. Microinjection into groups Cas9 and LATS2 occurred 10 hours after fertilization. Embryos were evaluated at day 4 of culture (D4) for assessment of cleavage rates (cleaved/total oocytes) and at D8, for assessment of blastocyst (blastocysts/total oocytes) and development (blastocysts/cleaved) rates. Embryos injected with LATS2 gRNA were stained with 1mg/ml Hoechst 33342 (Sigma) to verify if they have surpassed the 16-cell stage. Only mean rates and respective standard deviations are reported due to lack of degree of freedom to perform statistical analyses yet. The results of three replicates show that for cleavage, blastocyst and development rates control group presented 58 ± 3.90%; 30 ± 1.95% and 52 ± 5.57%, the Cas9 group presented 36 ± 5%; 10 ± 7% and 31 ± 25%, while the LATS2 group presented 50 ± 6.88%, 6 ± 1.12% and 12 ± 2.9%, respectively. Embryos in LATS2 group that did not develop to the blastocyst stage showed more than 16 cells after nuclear staining. These preliminary results suggest that injection of CRISPR/Cas9 along with LATS2 gRNA inhibited the formation of blastocysts without blocking embryonic development at earlier stages. Financial support by FAPESP, grants no. 2017/09576-3, 2017 / 25574-0 and 2018/18924-8.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Effect of heat stress during oocyte de novo DNA methylation on mice oocyte developmental competence

**Caroline Alencar Imaeda de Carvalho[1], Marcelo Tigre Moura[1], Daniela Bebbere[2], Francesca Mossa[2], Flávia Regina de Oliveira Barros[3], Fabíola Freitas Paula-Lopes[1]**

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There is a great amount of evidence indicating that mammalian growing oocytes are susceptible to heat stress (HS). From an epigenetic point of view, oocyte growth is of great importance due to *de novo* DNA methylation. Therefore, the objective of this study was to establish a mouse model to target heat stress during the first wave of *de novo* DNA methylation on oocyte developmental competence. C57BL/6 and CD1-Swiss pups were randomly allocated to heat stress (35°C for 12 h during the light period followed by 21°C for 12 h during the dark period; n= 12 CD1-Swiss and n= 11 C57BL/6) or control (21°C for 24 h; n=5 CD1-Swiss and n= 6 C57BL/6) from postnatal day 10 (P10) to 21 (P21) - the window of *de novo* DNA methylation. HS females were allocated under control temperature (21°C) from P22 until puberty (P35). At P35 animals were subjected to superovulation (10 I.U. intraperitoneal eCG followed by 10 I.U. of hCG with 48 h interval). Euthanasia was performed 12–14h after hCG and cumulus-oocyte complexes (COCs) were recovered from the oviducts of CD1-Swiss (n= 269 COCs for control and n= 160 for HS) and C57BL/6 (n= 101 COCs for control and n= 252 for HS). COCs were denuded, selected and subjected to parthenogenetic activation using calcium-free M16 medium (Sigma) supplemented with 10 mM strontium chloride, 5 µg/mL cytochalasin B, and 0.1% polyvinylalcohol at 37°C under 5% $CO_2$ for 5h. Activated oocytes were cultured in M16 medium for 4 days. The percentage of oocytes that cleaved and developed to morula and blastocyst stages was determined at 24 and 96 h after the beginning of activation, respectively. Data were subjected to analysis of variance using the General Linear Models procedure of SAS. There was no effect of heat stress on morphological oocyte viability regardless of mice strain. The effect of heat stress on cleavage rate and preimplantation embryonic development was affected by temperature x strain interaction. Heat stress did not affect CD1-Swiss cleavage rate (89.5 $\pm$ 4.5% versus 81.2 $\pm$ 6.6% for control and HS, respectively). However, HS blocked (P< 0.001) CD1-Swiss preimplantation development to morula and blastocyst stages. While 23.7 + 7.9% of the oocytes collected from control mice developed to morula/blastocyst stage after activation, none of the oocytes collected from HS mice did. In contrast, C57BL/6 oocyte developmental competence was very low regardless of temperature indicating the standard activation protocol used would need to be optimized for this mice strain. In conclusion, exposure of CD1-Swiss female mice to heat stress during the first wave of *de novo* DNA methylation fully compromised oocyte developmental competence. Support: FAPESP 2017/20125-3.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Less is more: Reduction in nutrient availability during in vitro culture is beneficial for bovine embryo development

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The *in vitro* production (IVP) systems relies on replicating the conditions of the female reproductive tract in order to generate blastocysts more alike those produced *in vivo*. However, the availability of nutrients in a dynamic system, such as the *in vivo*, may be different from that required for a static *in vitro* system. Our group previously demonstrated that reduced amounts of nutrients in culture medium produced embryos with distinct metabolic characteristics associated with an increase in embryo production (Santos et al, Anim. Reprod. 16(3):669, 2019). In this work, we present additional data regarding the effect of this reduction on embryonic metabolome. Bovine embryos were produced *in vitro* by conventional protocols and divided in 2 experimental groups: (i) ECS 100 - embryos cultured in Embryonic Culture Supplementation (ECS), a sequential serum-free media based on the composition of bovine oviduct (ECS100-Ov) and uterus (ECS100-Ut) fluids in terms of energy substrates (glucose, pyruvate, lactate) and amino acids; (ii) ECS 50 – embryos cultured in 50% of substrates present in ECS100. At the time of IVC, presumptive zygotes were transferred to ECS100-Ov or ECS50-Ov. At Day 4 (D4), embryos were transferred to their correspondent ECS100-Ut or ECS50-Ut where they remained until Day 7 (D7). For metabolome assessment, 5 blastocysts from each group were transferred to 50µL drops of their respective ECS-Ut for 12 hours (D7). Then culture media were collected and submitted to Raman spectroscopy analysis (Santos et al., BOE 6(8):2830-2839, 2015). Data were collected in 4 replicates and submitted to multivariate (PCA – Minitab program) and univariate (T-test) analysis ($p<0.05$; GraphPad Prism software). Peak attribution was performed according to previous report (Santos et al., Metabolomics (12):94). Raman analysis revealed twenty-nine metabolites mostly related to DNA, RNA, amino acids, proteins and lipids compounds. From those, twenty-one were differentially represented between groups; four of them were upregulated in ECS 50 and seventeen upregulated in ECS100. Metabolites upregulated in ECS100 group were related to DNA (Thymine, Adenine, Guanine), RNA, amino acids (Arginine, Threonine or Tryptophan), protein (amide I and II), glycogen and cytochrome C. Metabolites upregulated in ECS50 group were related to proteins, lipids and phospholipids. The literature highlights that the ideal culture medium should help the embryos carry out their developmental program with the minimum amount of energy, promoting a quieter metabolism instead of an overactive metabolism (Leese, Bioessays 24(9):845-9, 2002). Thus, our data suggest that in fact “less is more” once ECS50 may provide a quieter metabolism, therefore more suitable conditions for the development of viable and healthier bovine embryos. Acknowledgment: Fapesp (2017/18384-0; 2016/00350-0), CEM-UFABC.

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Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Effect of chromatin modifiers on histone and DNA methylation of bovine embryos derived from heat-shocked oocytes

**Clara Slade Oliveira[1], Carolina Capobiango Romano Quintão[1], Naiara Zoccal Saraiva[1], Luiz Gustavo Bruno Siqueira[1], Carolina David[1], Alex Cabral Vieira[2], Luiz Sergio Almeida Camargo[1]**

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Heat shock (HS) has a negative impact on oocyte competence and can lead to alterations on chromatin organization of embryos derived from those oocytes. Scriptaid and 5-aza-2'-deoxycitidine (5AZA) are chromatin modifiers that have been shown to favor chromatin remodeling of somatic cell nuclear-transferred embryos (Ding et al., Theriogenology, 70: 622-30, 2008; Wang et al., Cell Reprogram, 13: 431-39, 2011). This study aimed to evaluate whether such chromatin modifiers would influence the chromatin organization of early embryos derived from heat-shocked oocytes. For that, levels of histone (H3K9me3) and DNA (5-methylcytosine) methylation were assessed in 8-16 cells embryos from heat-shocked oocytes. Immature oocytes were randomly distributed in control (non-heat shock) and HS groups. In vitro embryo production was performed according to procedure reported elsewhere (Camargo et al., Cryobiology, 63: 256-62, 2011). HS was carried-out by exposing oocytes to 41.5°C for 12h plus 38.5°C for 12h during IVM. Presumptive zygotes were exposed to 500 nM Scriptaid or 100 nM 5AZA for 0h or 24h, composing the following groups: Control (C); Heat shock (HS); C+5AZA; HS+5AZA, C+Scriptaid and HS+Scriptaid. Embryos with 8-16 cells at 44h post in vitro-fertilization (hpi) were fixed and immunostained with anti-H3K9me3 (1:100, Merck 07-442) and anti-5-methylcytosine (5mC; 1:100; Merck MABE146) primary antibodies, following protocols reported elsewhere (Camargo et al., Reproduction, 158: 313-22, 2019; Sangalli et al., Cell Reprogram, 14: 235-47, 2012). Immunofluorescence was evaluated by epifluorescence microscopy and images processed by ImageJ. Corrected fluorescence was calculated for every nucleus (299 and 363 nuclei for 5mC and H3K9me3, respectively). Blastocysts rate was recorded at 192 hpi. Developmental data was analyzed by logistic regression and fluorescence data by Kruskal-Wallis with Dunn's test. Blastocyst rate was higher ($P<0.05$) in C (38.1±5.2%) than in other groups (HS: 17.1±4.5%; C+5AZA: 22.8±2.6%; HS+5AZA: 16.4±3.1%; C+Scriptaid: 24.8±4.9% and HS+Scriptaid: 15.2±3.8%). H3K9me3 levels were not different ($P>0.05$) between C (27.3±1.0) and HS (32.3±2.4) but it was higher ($P<0.05$) in C+5AZA (33.9±1.6) than in C. However, 5AZA did not affect H3K9me3 in embryos from heat-shocked oocytes (HS vs HS-5AZA, $P>0.05$). H3K9m3 level was similar ($P>0.05$) between C and C+Scriptaid; however, it was lower ($P<0.05$) in HS+Scriptaid (24.4±1.2) than in HS (32.3±2.4) group. DNA methylation was not affected ($P>0.05$) by heat shock during IVM or by exposure to Scriptaid or 5AZA. Despite no effect of heat shock on H3K9me3 level was found, we reported that histone methylation in embryos from heat-shocked oocytes can display responses to chromatin modifiers distinctly from embryos derived from oocytes matured under thermoneutral temperature, suggesting that chromatin of such embryos may exhibit an altered susceptibility to external agents. Financial support: Fapemig, Faperj, CNPq.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# MGA for synchronizing estrus behavior: a novel approach in ex situ South American deer species reproductive management

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The most common estrus synchronization in domestic ruminants is based on CIDR intravaginal device, yet in non-domestic ruminants, this device is considered a bit stressful due to the need for physical or/and chemical restraint. In this respect, other non-invasive methods are required, such as the estrus synchronization by means of oral progestogens e.g. melengestrol acetate (MGA). MGA is a synthetic progestogen that is used for estrus synchronization in domestic animals, however in wildlife species (e.g. deer), as far as we know it is used for contraceptive purposes. The main goal of this study was to assess MGA effectiveness in controlling estrus in red brocket deer (*Mazama americana*) females and verify corpus luteum formation after the synchronization protocol. Four red brocket deer females were used. Every female received an application of 1mg/ml IM of estradiol benzoate on day -8 (D-8). A dose of 2mg/animal/day of MGA® Premix fractionated in two administrations (1 mg/morning and 1 mg/afternoon) mixed with a banana, from D-8 to D-1 and a prostaglandin injection in Day 0**.** After MGA treatment, estrous behavior (EB) was evaluated 2 times per day and faecal collection started on D1 for corpus luteum (CL) evaluation. CL was assessed by faecal progesterone metabolites (FPM) measurement using competitive progesterone enzyme immunoassay (EIA). EB was observed between 24 to 72 hours (D1~D3) after prostaglandin injection for all females. Due to different times of EB in each female, we defined the last day of each EB as 'Day after EB (DAEB)' and compared the values from DAEB, with subsequent days (total days evaluated = 8). For determining the value indicative for CL formation, we took the lowest FPM value, which corresponds to the day after EB manifestation and when the value fell above a criterion value (mean DAEB+2SD)**,** it was considered an FPM rise. FPM raised three days after the last day of EB for one female and in four days after the last day of EB for other females**,** reaching values from 569 to 719 ng/g respectively, which values are close to those of the luteal phase of red brocket deer (716.6–8365.4 ng/g). Although we did not assess the fertility of the MGA protocol, the inhibition of the EB during the period of MGA treatment and FPM rise after EB might indicate that the synchronization protocol based on MGA treatment in red brocket deer is effective and it can be used as a potential estrus synchronization protocol in captive deer species. This work was supported by CNPq (Brazilian National Research Council).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

## Heat stress affects mouse ovulation rates and oocyte DNA methylation

**Marcelo Tigre Moura[1], Caroline Alencar Imaeda Carvalho[1], Flávia Regina Oliveira Barros[2], Francesca Mossa[3], Daniela Bebbere[3], Fabíola Freitas Paula-Lopes[1]**

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The follicle-enclosed oocyte is very susceptible to heat stress (HS). There is evidence that HS affects the series of cellular and molecular events triggered during oocyte growth compromising oocyte maturation and embryonic development. Genome-wide DNA methylation reprogramming is a major event that takes place during oocyte growth and early preimplantation embryonic development. Changes in the epigenome may reflect exposure to environmental stressors, although there is limited understanding of such effects during oogenesis. Therefore, the aim of this study was to develop a mice model to target HS during the first wave of de novo DNA methylation. CD1-Swiss lactating females and pups (F0 progeny) were randomly allocated to HS (35°C for 12 h / light period; 21°C for 12 h / dark period; n = 4 litters) or control (21°C for 24 h; n = 2 litters) from postnatal day 10 (P10) to 21 (P21). Following 11 days of HS, females were allocated under control temperature (21°C) until puberty (P35). The pups were weighted on days P10, P15, P21 (weaning) and gender-matched littermates were weighted weekly until P35 for females (n = 45) and P84 for males (n = 27). At puberty (P35) F0 females were superovulated with intraperitoneal injections of 10 IU eCG and 10 IU hCG with a 44-46 h interval. Oviducts were dissected 12-14 h post-hCG. Cumulus-oocyte complexes were harvested from oviducts in M2 medium under a stereomicroscope. Mature oocytes were denuded in 0.1% hyaluronidase and washed in M2 medium to determine ovulation rates. DNA methylation analysis relied on indirect immunofluorescence. Oocytes were fixed in 4% (w/v) PFA for 20 min. Oocytes were permeabilized in 0.5% (v/v) triton X-100 for 30 min and incubated with 75 µg/mL RNAse A for 1h. DNA denaturation was attained by incubation in 4N HCl for 30 min and Tris-HCl for 30 min. Oocytes were blocked overnight in 10% (v/v) goat serum solution at 4ºC. DNA methylation was detected with 5-methylcytosine monoclonal antibody (Cell Signaling; 1:1,000 dilution) for 1h. Oocytes were incubated with anti-goat secondary antibody Alexa Fluor 488 (Invitrogen; 1:200 dilution) for 1h. DNA staining was carried out with 50 µg/mL propidium iodide for 15 min. DNA methylation quantification was performed with ImageJ. The data was subject to ANOVA with a significance level of 5%. Pup pre-weaning survival was similar between groups. There was no effect of HS on pup weight on P15 (CTL: 8.64±1.53g vs. HS: 9.17±1.41g) and P21 (CTL: 12.70±2.39g vs. HS: 12.92±1.99g). The post-weaning weight of both genders was not affected by HS. The ovulation rates (number of ovulated oocytes/number of females) were reduced in HS (12.8±3.4) compared to control (26.5±3.7; P = 0.013). Remarkably, oocyte DNA methylation was increased 1.49 fold in HS oocytes as compared to control (CTL n = 29 oocytes; HS n = 25 oocytes; P < 0.0001). In conclusion, HS during oocyte growth reduces ovulation rates and leads to oocytes with aberrant DNA methylation levels. Support: FAPESP 2017/20125-3.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Negative energy balance modulates ISG15 expression in the endometrium during maternal recognition of pregnancy in ewes

**Gabrielle Rebeca Everling Correa, Igor Gabriel Zappe, Carolina do Santos Amaral, Manuela Wolker Manta, Karine de Vargas Aires, Alessandra Bridi, Valério Marques Portela, Alfredo Quites Antoniazzi**

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Embryonic mortality during maternal recognition of pregnancy is a major cause of reproductive failure in ruminants. The negative energy balance (NEB) is one of the causes of reduced fertility. The aim of our study was to evaluate the effects of NEB on early pregnancy in sheep, more specifically on abundance of mRNA encoding *ISG15* in the endometrium on Day 17 of pregnancy. For this, 21 crossbred Texel-Corriedale ewes had estrus and ovulation synchronized. The ewes were distributed into four experimental groups: non-pregnant, non-bred control group (CNP, n=3); pregnant control group (CP, n=6); NEB group during the first week of embryonic development (FW; n=6); and NEB group during the second week of embryonic development (SW; n=6). There ewes were receiving a diet based 1.5 kg of tifton85 hay and 0.25 kg of cracked corn per day for 30 days to allow adaptation to experimental conditions. The animals had unrestricted access to water and mineral supplement. The experimental groups received this same diet during the experimental period, however FW group fasted from Days 0 (Breeding day) to 7 and SW group fasted from Days 9 to 16 for the induction of NEB with hyperketonemia. The blood β-hydroxybutyrate (BHBA) levels and glycemia were measured. On Day 17 of the estrous cycle or gestation, ewes were euthanized and endometrium was collected ipsi and contralateral horns to ovulation. The relative mRNA *ISG15* abundance was assessed by q-PCR. Body condition score (scale of 1–5) were determined, on day 0 were 3.50±0.25 (CNP), 3.46±0.56 (CP), 3.60±0.52 (FW), 3.12±0.32 (SW), on day 7 were 3.50±0.25 (CNP), 3.46±0.56 (CP), 3.10±0.42 (FL), 3.25±0.35 (SW) and on day 17 were 3.25±0.25 (CNP), 3.63±0.54 (CP), 3.15±0.38 (FW), 2.87±0.32 (SW). Age (months) were for CNP (24± 10.39), CP (24±14.70), FW (21.6±9.10), SW (25.5±15.78). Fasted ewes had strong negative correlation (R2=0.72; p<0.001) between glycemic levels and fasting time in the first two days of fasting, there was a decrease in glycemia from 55.78±6.31 (on Day 0; n = 9) to 30.00±1.65 (on Day 2; n=9). Fasted ewes had moderate positive correlation (R2=0.60; p<0.001) between blood BHBA levels and fasting time on the seven days of fasting, there was an increase in blood BHBA levels of 0.24±0.03 (Day 0; n = 9) to 0.89±0.18 (Day 7; n=9). Pregnant ewes (2.81±0.27) had greater mRNA *ISG15* expression in the endometrium ipsilateral horn when compared to non-pregnant ewes (1.01±0.12) ipsilateral horn (p<0,05). The CP (3.14±0.55) group had greater expression of *ISG15* than the CNP (3.14±0.12) group in the endometrium from ipsilateral horn. The FW (2.58±0.41) and SW (2.29±0.33) groups had no difference on *ISG15* mRNA levels comparing to the CNP (1.01±0.12) and CP (3.14±0.55) groups in the contralateral endometrium. In conclusion, ewes that had periods of NEB during the second week of embryonic development, express a lower level of *ISG15* mRNA in the ipsilateral endometrium comparing to pregnant ewes that did not have NEB.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Conjugated linoleic acid supplementation reduces prostaglandin E2 and F2a secretion from bovine trophoblastic cells in vitro

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Early embryonic mortality represents a major cause of reproductive failure in cattle. Strategies to decrease synthesis of prostaglandin $F_{2alpha}$ ($PGF_{2a}$) and increase synthesis of prostaglandin $E_2$ ($PGE_2$)can benefit the establishment of pregnancy. Conjugated linoleic acid (CLA) supplementation in cell culture medium affectsthe synthesis of prostaglandins; however the effect of CLA supplementation on cultured bovine trophoblast cells (CT1) has not been determined. The hypothesis of this study is that CLA supplementation on *in vitro* culture medium of CT1 cells increase synthesis of $PGE_2$ and decrease synthesis of $PGF_{2a}$, benefiting the establishment of pregnancy. The aim was to determine the effects on $PGE_2$ and $PGF_{2a}$ synthesis with supplementation of varying concentrations of CLA (Sigma-Aldrich, USA, Cat N°. O5507) on *in vitro* culture of CT1 cells. The CT1 cells were cultured for 22 days in a humidified incubator at 38.5□C with 5% $CO_2$ until they reached 100% confluence. On the 23th day they were transferred to six-well plates with DMEM (1X) + GlutaMAX medium supplemented with 10% of fetal bovine serum (FBS), 1% of non-essential amino acids, 1% of antibiotic-antimycotic and 0.001% of □-mercaptoethanol, to be cultured for another 5 days until reaching 50% confluence. Twenty-four hours before CLA supplementation (day 6 of culture), the medium was replaced with a new medium without FBS, and on day 7 medium without FBS was supplemented with varying CLA concentrations (0, 10, 20, 50 or 100 µM) for a 24-hour culture period. Collected medium was stored at -20□C until analysis. A total of five culture replicates were performed. Concentrations of $PGE_2$ and $PGF_{2a}$ on day 8 were determined by enzyme-linked immunosorbent assay. Statistical analyzes were performed using the PROC MIXED of SAS program (version 9.2, SAS Institute Inc., Cary, NC, USA) considering the main effect of treatment group and the random effect of culture replicate. Concentration of $PGE_2$ was greater ($P$ = 0.04) for control (89.74 ± 4.39 ng/mL) in comparison to 10, 20, 50 and 100 µM of CLA (63.65 ± 8.51; 56.28 ± 9.66; 58.61 ± 9.31 and 64.77 ± 11.59 ng/mL, respectively). Concentration of $PGF_{2a}$ was also greater ($P$□ 0.0001) for control (66.67 ± 7.89 ng/mL) in comparison to 10, 20, 50 and 100 µM of CLA (33.49 ± 5.01; 24.86 ± 4.41; 26.22 ± 4.53 and 25.73 ± 3.23 ng/mL, respectively). A significant effect ($P$ = 0.0007) on $PGE_2/PGF_{2a}$ ratio was also observed, reflecting a greater ($P$< 0.05) ratio in CLA-treated groups (1.99 ± 0.21; 2.39 ± 0.31; 2.35 ± 0.26 and 2.55 ± 0.39, respectively) compared to the control group (1.41 ± 0.13) and a greater ($P$< 0.05) ratio in CTl cells treated with 100 µM compared to 10 µM of CLA. We conclude that CLA treatment for 24 hours on *in vitro* culture medium of CT1 cells decreased $PGE_2$ and $PGF_{2a}$ synthesis, but a CLA dose-dependent effect was observed on $PGE_2/PGF_{2a}$ ratio. Acknowledgement:grant #2018/24168-1 and #2019/00637-5, São Paulo Research Foundation (FAPESP).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Role of 300 IU of human chorionic gonadotropin administrated seven days after estrus onset in dairy toggenburg goats

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This study assessed the role of hCG administrated on Day 7 (D7) after estrus onset in dairy goats during the transition season. Toggenburg female goats received an intravaginal sponge containing 60 mg of medroxyprogesterone acetate (Progespon®; Syntex S.A., Argentina) for 6 d plus 200 IU of eCG (Folligon®; Intervet International B. V., Holanda) and 30 µg of d-cloprostenol (Prolise®; ARSA S.R.L., Argentina) i.m. 24 h before sponge removal. The females were hand-mated with fertile bucks (1:6 male/female ratio) for 96 h, at 24 h interval while in estrus (two mating in maximum). On D7, the animals received either 1 mL of saline (control, n=43) or 300 IU of hCG (hCG group, n=43) i.m. Transrectal ultrasound (US) exams were performed on D7, D13, D17 and D21. The P4 concentrations were determined in 25 goats (control: n=12; hCG: n=13) at the same days of US exams. Data were analyzed by three-way ANOVA, considering the main effects of Group, Pregnancy status, Day of observation or their interactions (P<0.05). The hCG group (90.7%; 39/43) had a greater (P<0.05) pregnancy rate compared to control (74.4%; 32/43). A total of 46.5% (20/43) of the animals in the hCG group developed accessory luteal structures (ALS). Within the hCG group, the final number of all luteal structures [ovulatory corpora lutea (CL)+ALS] in D21 was greater (P<0.05) than in D7 (2.1±0.1 compared with 1.6±0.1, respectively). All hCG goats that had ALS and 82.6% of goats without ALS post-treatment remained pregnant. There was an increase (P<0.05) in the total luteal area (TLA) from D7 to D13 in pregnant females from both groups. In hCG group there was a significant increase in pregnant compared to non-pregnant in D21. The mean vascular area (MVA) declined (P<0.05) by D21 in all non-pregnant does. In control, MVA was greater (P<0.05) in pregnant than in non-pregnant in D21. In hCG group, pregnant had greater (P<0.05) MVA than non-pregnant in D17 and D21. Serum P4 concentrations were similar (P>0.05) between groups in D13, but they increased (P<0.05) on D21 in pregnant does of both groups, and declined to a basal or non-detectable level in 5/43 (11.6%) before D21 in control, and 1/43 (2.3%; P<0.10) in the hCG group. In control, pregnant females had lower (P<0.05) P4 concentrations in D17 than in D7. Mean daily numbers of small and medium-sized antral follicles decreased (P<0.05) only in pregnant of both groups with a decline in medium follicle numbers occurring earlier in hCG (D13) compared with control (D17). We concluded that a single dose of hCG given on D7 after estrus significantly increased the pregnancy rate in synchronous estrous-induced Toggenburg goats by a mechanism that was at least partly independent of luteal tissue content/vascularity and P4 secretory ability. Financial Support: CNPQ (Project 314952/2018-7), Fapemig (Project CVZ-PPM 00201-17) and EMBRAPA (Project 20.19.01.004.00.03.001).

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Feeding a mycotoxin adsorbent (Notox-LS®) can avoid detrimental effects of zearalenone on cattle oocytes

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Objectives of this study were to test whether addition of a mycotoxin adsorbing agent could block detrimental effects of feeding diets contaminated with zearalenone to heifers on *in vitro* embryo production. Nelore heifers (n = 30), weighing 350 ± 3,3 kg and aging 18 ± 0,2 mo received same basic diet composed (DM/day) of 70% forage and 30% concentrate (corn meal 550g/kg, gluten feed 200g/kg, soybean meal 200g/kg, urea 10 g/kg, and mineral supplement 40g/kg), balanced to attain 1kg of body weight gain per animal/day. Animals were randomly assigned to 3 dietary treatments, as follows: 1) Control (CON): no contamination with zearalenone, n = 10; 2) Zearalenone (ZEA): concentrate portion of the diet contaminated with zearalenone (aiming total intake of 300ppb of zearalenone/heifer/day), n = 10; 3) Zearalenone (300ppb/animal/day) supplemented with 20g/animal/day of a mycotoxin adsorbing product Notox-LS, Cargill Animal Nutrition and Health (ZEA+ADS), n = 10. Heifers included in each treatment were fed in separate pens once/day and kept on the same feeding regimen for a total of 12 weeks and then slaughtered. Upon slaugthering, ocytes were retrieved and graded according to IETS guidelines by the same technician. Briefly, viable oocytes were placed in a maturation media upon retrieval, and later fertilized with the same semen batch from one fertility-proven bull to minimize sire variation. Each fertilization droplet received $1x10^6$ live sperm cells/mL, and at approximately 18 h after insemination, the cumulus cells were transferred to 100 µL drops of embryo culture medium according to a standard IVF procedure. Seven days later structures were graded by the same technician. Data was analyzed with the proc GLIMMIX of SAS, and amount of oocytes produced per donor was included as a covariate in the statistical model, as well as “heifer”, as a random effect in all models. There were no differences in body weight gain or feed intake across groups throughout the trial period. ZEA contamination significantly reduced ($P < 0,05$) the proportion of viable oocytes (49,4%, n = 207) compared to uncontaminated diets CON (59,9%, n = 222) and the addition of a mycotoxin adsorbent agent seemed to restore oocyte quality in heifers receiving a contaminated diet ZEA+ADS (63,8%, n = 172). Moreover, once good quality oocytes were placed in an embryo culture that was not contaminated with zearalenone, blastocyst rate did not differ among Experimental groups (CON = 17,0%; ZEA = 21,7%; ZEA+ADS = 18,1; $P > 0.10$). In conclusion, these findings highlight the potential detrimental effects at cellular level on reproductive physiology in cattle exposed to mycotoxins, and that the use of a mycotoxin adsorbing agent can counteract some of these detrimental effects. Future research, utilizing both *in vivo* or *in vitro* models, and differing levels of contamination with zearalenone after oocyte fertilization are needed to further evaluate its effects on embryo development.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Embryology, developmental biology, and physiology of reproduction

# Oocyte maturation with slim serum decreases lipid storage without comprising embryo development

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Lipids play crucial roles in many biological processes including membrane formation, cell signaling, energy storage and metabolism. However, serum lipids present in the culture medium may be internalized by oocytes and embryos during IVP and accumulated in lipid droplets, leading to abnormalities in energy metabolism, gene expression and epigenetics programming. In this study, we explored how the removal of lipid compounds present in fetal bovine serum (FBS) would impact *in vitro* development and the lipid profile of oocytes and resulting blastocysts. For this purpose, we used organic solvents to remove nonpolar lipids present in FBS (neutral lipids like triacylglycerol, TAG; cholesteryl esters; CE and free fatty acids; FFA). The remaining extract (*slim serum* containing polar metabolites) was resuspended in IVM medium (TCM-199 bicarbonate with hormones, pyruvate and antibiotics). Then, bovine COCs obtained from abattoir ovaries were submitted to IVM in 2 groups: OS (IVM medium + 10% whole FBS) and OSS (IVM medium + 10% *slim serum*). After 24 h, matured oocytes were collected (n=10/group); and those remaining in each group underwent IVPE by standard protocols under the same culture conditions. At Day 7, production rates were assessed, and expanded blastocysts were collected (BS and BSS; n=10/group). Finally, lipid extracts from individual oocytes/embryos were analyzed by Multiple Reaction Monitoring (MRM)-Profiling mass spectrometry. Using a broad screening, we investigated 379 lipids from 10 classes (TAG, CE, FFA, acyl-carnitines, sphingomyelins, SM; and phospholipids derived from choline, PC; ethanolamine, PE; glycerol, PG; serine, PS; and inositol, PI). Exploratory analysis was performed by principal component analysis (PCA; Metaboanalyst 4.0); fold-change (FC) values were calculated based on the relative abundance of lipid ions (FC>2 and p<0.05). IVP rates were compared by t-test (□=5%). PCA revealed that oocytes matured in whole or *slim serum* have very distinct lipid profiles, with 163 lipids statistically different between groups. Oocytes matured in whole serum show higher abundances of TAG and CE, most of them are at least 5 times higher in group OS. Meanwhile, group OSS shows higher amounts of FFA and some phospholipids classes (PC, SM, PE, PG). We did not observe significant differences in blastocyst rates between BS and BSS, however, their lipid profile was also very distinct. BS group showed up to 50 times increase in some TAGs, while BSS presented an enrichment for phospholipid classes (PC, SM and PS) as well as for 80% of all analyzed FFA (up to 80-fold increase in palmitic acid). In conclusion, the *slim serum*, which contains mostly metabolites (amino acids, nuclei acids sugars, small organic acids and polar lipids) was still able to support maturation. Nonetheless, it substantially altered the lipid profile, originating oocytes and blastocyst with relatively less storage lipids, which may be of great importance for cryopreservation.