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Effect of Hormonal Supplementation Periods and *In Vitro* Maturation Media on Developmental Competence of Pig Oocytes

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ABSTRACT

***Background*:** The oocyte ability to undergo successful fertilization, cleavage and embryonic development depends on meiotic maturation and developmental competence acquisition. *In vitro* maturation (IVM) protocols currently use eCG, hCG or a combination of both, the effect of these gonadotrophins during IVM and subsequent embryonic development is still controversial. Several media have been used for IVM of porcine oocytes: TCM199, Whitten's and NCSU23 have also been shown to support pig oocyte IVM. This study was designed to determine the effect of hormonal supplementation period and maturation media during *in vitro* maturation of pig oocytes (1) and subsequent embryonic development (2).

***Materials, Methods & Results*:** Oocytes with intact *cumulus oophurus* layers and homogeneous cytoplasm were collected from prebubertal gilts. IVM was subjected in NCSU23, TCM199 or Whitten's media supplemented with 10 IU/mL eCG and 10 IU/mL hCG for the first 24 or 48 h of IVM. In each replicate the oocytes were fixed every 4 h from 32 to 48 h IVM or the past 48 h after IVM, oocytes were fertilized *in vitro* in mTBM medium for six hours and cultured in NCSU23 medium for nine days. Cleavage, blastocyst and hatching rates were evaluated at 48 h (day 2), 168 h (day 7) and 216 h (day 9), respectively.

The addition of eCG and hCG during the first 24 h IVM increased the proportion of oocytes that reached MII stage at 44 h of maturation in NCSU23 medium. This effect was also observed in Whitten medium at 44 and 48 h (P <0.05). However, it was not observed in the TCM199 medium. No effect of maturation medium on oocyte nuclear maturation (P >0.05) was observed in oocytes matured in the presence of eCG and hCG during the first 24 h IVM or during 48 h IVM. A progressive increase of maturation indexes was observed on oocytes matured with hormonal supplementation in Whitten media for 24 h. Higher indexes were obtained at 44 and 48 h. When NCSU23 media was used, no difference after 36 h of maturation was observed. The same result was observed in TCM199. A progressive increase of maturation indexes was observed on oocytes matured with hormonal supplementation for 48 h in Whitten media. Higher indexes were obtained in 36 and 40 h. When NCSU23 or TCM199 were used, no difference was observed. No effect of IVM media on the percentage of fertilized oocytes and polyspermic oocytes or number of spermatozoa per fertilized oocytes was observed. Also, no effect of IVM media on cleavage and blastocyst rates was seen. However, the proportion of hatched blastocysts was lower in NCSU23 compared to Whitten or TCM199.

***Discussion*:** Similar results were reported by Marques *et al.* [13], that it no differences between hormonal supplementation for 22 or 44 h were observed. Therefore, more studies are needed to elucidate the role of these hormones in nuclear *in vitro* maturation in pig oocytes.

In conclusion, no effect of maturation media on meiotic progression was observed. However, the proportion of oocytes that reached metaphase II (MII) stage was higher when eCG + hCG were added for 24 h than 48 h mainly at the 44 h of maturation. In addition, no differences were observed in cleavage and blastocyst rates of the cultured embryos. However, embryos cultured in NCSU23 showed lower rates of hatching compared to other media. These results indicated no effect of maturation media on the fertilization and embryonic development even in the presence of cysteine, PFF and EGF, except for hatched embryos that these rates were lower in NCSU23.

Keywords: swine, fertilization, polispermy, *in vitro* maturation, maturation media, hormonal supplementation.

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INTRODUCTION

Spontaneous nuclear maturation occurs when oocytes are removed from their follicular environment and are submitted to *in vitro* maturation. However, a small proportion of these oocytes reaches the blastocyst stage following *in vitro* fertilization (IVF). A strategy to increase the developmental ability of *in vitro* matured oocytes is the maturation medium supplementation with gonadotrophic hormones. Although most *in vitro* maturation (IVM) protocols currently use eCG, hCG or a combination of both, the effect of these gonadotrophins during IVM and subsequent embryonic development is still controversial [6].

Several media have been used for IVM of porcine oocytes [1]. Tissue culture medium (TCM)-199 is chosen by most laboratories [8,13]. Nevertheless, a modified Whitten's medium [8] and NCSU23 medium [9] have also been shown to support pig oocyte IVM.

Such differences among studies may be due to donor age, follicle size, media composition, hormone processing or follicular health status [3]. These factors can modulate positive or negative effects during IVM and embryo development.

The aim of the present study was to investigate the effect of three maturation media TCM199, Whitten or NCSU23 supplemented with eCG and hCG for 24 or 48 h on IVM of pig oocytes. In addition, the best hormonal treatment was chosen to examinate oocyte developmental capacity.

MATERIALS AND METHODS

Ovary collection, follicle aspiration, and selection of oocytes

Abattoir-derived porcine ovaries were transported to the laboratory at 25-28°C. Follicles between 2-5mm were aspirated with an 18 gauge needle attached to a 10 mL syringe and the follicular fluid was transferred to 50 mL conical tubes. Oocytes with intact *cumulus oophurus* layers and homogeneous cytoplasm were selected for IVM.

Experiment 1: *Effect of maturation media and hormonal supplementation period on nuclear maturation*

This experiment, presented as a 2 x 3 factorial design, evaluated the effects of maturation media (TCM199, NCSU23 or Whitten) and hormonal treatment periods (24 or 48 h of IVM). Cumulus-oocyte complexes (COCs) were ramdomly distributed in the following treatments: TCM199**,** NCSU23 or Whitten media supplemented with 3.05 mM glucose and 0.91 mM sodium pyruvate) supplemented with 10 IU eCG/ml and 10 IU hCG/mL for the first 24 h IVM or for the entire 48 h maturation period at 39°C in an atmosphere of 5% CO_2 in air and high humidity.

Evaluation of nuclear maturation

Oocytes were removed from the maturation medium at 32, 36, 40, 44 or 48 h of IVM to evaluate meiotic progression. Cumulus-oocyte complexes were denuded by incubation with 0.5% (w/v) trypsin in PBS without calcium and magnesium at 39ºC for 3 min and by repeated pippeting. Denuded oocytes were fixed in 10% Triton-X 100 in 3.7% (w/v) paraformaldehyde for one hour and permeabilized in 10% Triton-X 100 in PBS with 0.3% (w/v) BSA for another hour. Oocytes were mounted on glass slides using drop glycerol containing 10 µg/mL Hoechst 33342 and covered with coverslips. The samples were examined under Zeiss epifluorescence microscopy (maximum excitation of 365 nm and maximum emission of 480 nm) to evaluate nuclear maturation rates. Oocytes containing one polar body and a metaphase plate were considered mature.

Experiment 2: *In vitro fertilization and embryo culture*

This experiment was designed to evaluate the effects of maturation media (TCM199, NCSU23 or Whitten) for 24 h and subsequent development. The procedure of IVM was the same used in experiment 1, except for the period of supplementation (24 h). After IVM, oocytes were washed three times in mTBM medium [mTBM consisting of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2 2H_2O$, 20.0 mM Tris, 11.0 mM glucose, 5.0 mM sodium pyruvate, 1 mM theophilin, and 0.1% BSA]. Groups of 30 to 35 oocytes were transferred to a 95 µL droplet of mTBM medium under mineral oil. The sperm-rich fraction of a fertile boar ejaculate was collected by digital pressure with gloved hand. A semen sample of 25 mL was diluted (1:1) in Beltsville Thawing Solution (BTS) and maintained refrigerated (15-18 °C) for 18-22 h before use. The semen was warmed to 37°C, centrifuged at 200 x *g* for 3 min. The 2 ml top supernatant was collected, washed three times by centrifugation at 1200 x *g* for 3 min, ressuspended in PBS, supplemented with 0.1% BSA and centrifuged

at 1200 x *g* for 3 min. The sperm pellet was ressuspended in mTBM medium and the concentration was adjusted to 15 x 10^7 spermatozoa/mL. Oocytes were fertilized with 5 µL of sperm solution to a final concentration of 3 x 10^5 spermatozoa/mL at 39 °C in an atmosphere of 5% CO_2 in air. Six hours after fertilization, oocytes were washed four times to remove sperm, and transferred to 100 µL droplet NCSU23 of media containing 4 mg/mL BSA in four-well plates (Nunc) until evaluation.

Evaluation of in vitro fertilization and embryo development

Presumptive zygotes and blastocysts were harvested at 18 and 168 h post-insemination, respectively. Samples were mounted on glass slides, fixed in Triton X-100 in paraformaldehyde for one hour and and mounted in glycerol containing 10 µg/mL Hoechst 33342. Samples were evaluated using Zeiss epifluorescence (maximum excitation of 365 nm and maximum emission of 480 nm). Oocytes were considered penetrated when they had at least one swollen sperm head or a male pronucleus with an extruded second polar body and the corresponding sperm tail in the vitellus. Oocytes with more than two pronuclei were considered polyspermic. The number of penetrated spermatozoa per oocyte was recorded.

Cleavage, blastocyst and hatching rates were evaluated at 48 h (day 2), 168 h (day 7) and 216 h (day 9), respectively.

Statistical Analysis

Data were analyzed using the JMP software (SAS Institute Inc. Cary, NC). Differences in the proportions of maturation and fertilization rates were determined by chi-square and differences in the fertilization, polispermy, number of spermatozoa per fertilized oocyte and embryo development rates were determined by Tukey-Kramer HSD test for multiple comparisons. A P <0.05 value was considered statistically significant.

RESULTS

Experiment 1: *Effect of maturation media and hormonal supplementation period on nuclear maturation*

Each experiment was replicated five times. The effect of maturation medium and hormonal supplementation periods on nuclear maturation are shown in Tables 1, 2 and 3.

The addition of eCG and hCG during the first 24 h IVM increased the proportion of oocytes that reached MII stage at 44 h of maturation in NCSU23 medium. This effect was also observed in Whitten medium at 44 and 48 h (P <0.05). However, it was not observed in the TCM199 medium (Table 1).

No effect of maturation medium on oocyte nuclear maturation (P >0.05) was observed in oocytes matured in the presence of eCG and hCG during the first 24 h IVM or during 48 h IVM (Tables 2 and 3).

A progressive increase of maturation indexes was observed on oocytes matured with hormonal supplementation in Whitten media for 24 h. Higher indexes were obtained at 44 and 48 h. When NCSU23 media was used, no difference after 36 h of maturation was observed. The same result was observed in TCM199 (Table 2).

A progressive increase of maturation indexes was observed on oocytes matured with hormonal supplementation in Whitten media for 48 h (Table 3). Higher indexes were obtained in 36 and 40 h. When NCSU23 or TCM199 were used, no difference was observed.

Experiment 2: *In vitro fertilization and embryo culture*

The effect of maturation media on fertilization rate is shown in Table 4. No effect of IVM media on the percentage of fertilized oocytes and polyspermic oocytes or number of spermatozoa per fertilized oocytes was observed.

Embryos were evaluated at 48 h (day 2), 168 h (day 7) and 216 h (day 9) to determine differences among developmental rates (Table 5). No effect of IVM media on cleavage and blastocyst rates was seen. However, the proportion of hatched blastocysts was lower in NCSU23 compared to Whitten or TCM199.

DISCUSSION

The current study demonstrated that IVM of prepubertal gilts oocytes in NCSU23, TCM199 and Whitten media supplemented with eCG and hCG during the first 24 h IVM present higher nuclear maturation rates than entire the 48 h IVM period. Moreover, when oocytes were cultured in NCSU23, TCM199 and Whitten, no differences were observed comparing fertilization and developmental parameters, except for the hatching data, which was lower in NCSU23 derived oocytes.

Table 1. Effect of hormonal supplementation period (24 or 48 h) on oocyte nuclear maturation in different maturation media (NCSU23, Whitten or TCM199).

	NCSU23 n (%)		Whitten n (%)		TCM199 n (%)	
Oocyte evaluation time	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
32h	14/41 (34.1)	13/35 (37.1)	9/40 (22.5)	5/27 (18,5)	18/50 (36)	18/50 (36)
36h	24/37 (64.9)	17/34 (50)	26/54 (48.1)	17/30 (56,7)	25/37 (67.6)	14/31 (45.2)
40h	16/31 (51.6)	14/35 (40)	23/48 (60.5)	19/36 (52,8)	28/45 (62.2)	11/29 (38)
44h	34/47 (72.3)[a]	13/32 (40.6)[b]	27/39 (69.2)[a]	15/37 (40,5)[b]	24/42 (57.1)	18/32 (56.2)
48h	30/44 (68.2)	15/27 (55.6)	24/34 (70.6)[a]	10/30 (33,3)[b]	26/44 (59.1)	13/29 (44.8)

[a,b] Different lowercase letters at the same line indicate significant difference among hormonal exposure periods for the same maturation media ($p < 0,05$).

Table 2. Oocyte maturation rates between 32 and 48 h in NCSU23, Whitten or TCM199 media supplemented with eCG and hCG during the first 24 h.

Oocyte evaluation time	NCSU23 n (%)	Whitten n (%)	TCM199 n (%)
32h	14/41 (34.1)[A]	9/40 (22.5)[A]	18/50 (36)[A]
36h	24/37 (64.9)[AB]	26/54 (48.1)[B]	25/37 (67.6)[B]
40h	16/31 (51.6)[AB]	23/48 (60.5)[B]	23/48 (60.5)[B]
44h	34/47 (72.3)[B]	27/39 (69.2)[BC]	24/42 (57.1)[B]
48h	30/44 (68.2)[AB]	24/34 (70.6)[C]	26/44 (59.1)[B]

[A, B] Different capital letters represent significant difference in the same column (same maturation media).

Table 3. Oocyte maturation rates between 32 and 48 h in NCSU23, Whitten or TCM199 media supplemented with eCG and hCG during 48 h.

Oocyte evaluation time	NCSU23 n (%)	Whitten n (%)	TCM199 n (%)
32h	13/35 (37.1)	5/27 (18,5)[A]	11/32 (34.4)
36h	17/34 (50)	17/30 (56,7)[B]	14/31 (45.2)
40h	14/35 (40)	19/36 (52,8)[B]	11/29 (38)
44h	13/32 (40.6)	15/37 (40,5)[AB]	18/32 (56.2)
48h	15/27 (55.6)	10/30 (33,3)[AB]	13/29 (44.8)

[A, B] Different capital letters represent significant difference in the same column (same maturation media).

Table 4. Effect of maturation media on fertilization, polispermy and number of spermatozoa per fertilized oocyte (P <0.05).

Media	Nº of oocytes evaluated	Percentage of oocytes		Number of spermatozoa per fertilized oocyte (n)
		Fertilized [a] n (%)	Polyspermic [b] n (%)	
NCSU23	60	51 (85)	40 (78.4)	4.1
Whitten	65	51 (79.7)	36 (70.6)	3.5
TCM199	76	61 (80.3)	42 (68.8)	3.3

[a]Number of fertilized oocytes/total number of oocytes.
[b]Number of polyspermic oocytes/total number of fertilized oocytes.

Table 5. Effect of maturation media on cleavage, blastocyst formation and hatching from oocytes matured in the presence of hormones for with 24 h (P <0.05).

Media	N° of oocytes examined	Cleavage (%)	Blastocyst (%)	Hatching (%)
NCSU23	148	43/148 (29)	28/148 (18.9)	5/28 (17.8)[a]
Whitten	151	54/151 (35.8)	33/151 (21.8)	11/33 (33.3)[b]
TCM199	170	72/170 (42.3)	34/170 (20)	11/34 (32.3)[b]

Different superscript lowercase letters present statistical significant differences (P < 0.05).

Regarding the different hormonal treatments used in this study, these IVM results are in disagreement with those observed by Schoevers *et al*. [19], which did not show differences between hormonal supplementation during the first 22 h or entire maturation period (44 h). This might be due to the use of sow oocytes instead of oocytes from prepubertal gilts. It has been reported that oocytes from sows have higher maturation potential than oocytes from prepubertal gilts [15].

Gonadotrophins withdraw from IVM medium after 24 h accelerated meiotic progression, resulting in more oocytes that reached the MII stage by 48 h. However, this result is controversial, since it was observed that the presence of eCG and hCG during the first 20 h, or during the entire maturation period of 40 h for oocytes from prepubertal gilts resulted in the same proportion of MII oocytes [9]. Similar results were reported by Marques *et al*. [13] and no differences between hormonal supplementation for 22 or 44 h were observed. Therefore, more studies are needed to elucidate the role of these hormones in nuclear *in vitro* maturation in pig oocytes.

Although the physiological signal that triggers the resumption of meiosis in prophase I-arrested oocytes is not well understood, some possibilities have been proposed. An alternative to explain the oocyte resumption is that cumulus cells produce a meiosis re-initiation signal in response to gonadotrophin stimulation [5,16]. A group of dimethylsterols designated meiosis-activating sterols (MAS) is secreted by cumulus cells in response to FSH stimulation [4]. Recently, MAS has been isolated from porcine follicular fluid (PFF) and considered capable to induce resumption of meiosis in prophase-arrested oocytes. Faerge *et al.* [7] showed that the addition of MAS to the maturation medium reduced the polyspermy rate. This suggests that MAS may play a role in cytoplasmic maturation, possibly leading to improved efficiency of the zona pellucida block. It has been previously demonstrated that MAS has a positive effect on cytoplasmic maturation of mouse oocytes leading to an improved fertilization rate [12].

These data are in accordance to the results of this study, in which media was supplemented with PFF. Probably after delay of resumption with

gonadotrophins, the presence of MAS was important to support adequate conditions, mainly when gonadotrophins were suppressed after 24 h of IVM. Moreover, the main regulator of the oocyte meiotic cell cycle is cyclic adenosine monophosphate (cAMP). *In vivo*, gonadotrophins trigger a transient increase in oocyte cAMP content, which subsequently decreases to initiate germinal vesicle breakdown (GVBD) [13]. Shimada *et al.* [18] reported that the cAMP content of prebubertal oocytes increases at 4 h IVM, reaches a peak level at 8 h, and then dramatically drops at 12 h and again at 28 h to reach basal levels at 32 h and remains low until the end of maturation. Sasseville *et al.* [17] observed a peak of cAMP at 13 h and the meiosis resumption occurred after 20 h. They concluded that the regulation of GVBD occurs depending on transient concentration of cAMP during first half of IVM.

Regarding hormonal treatment for 24 or 48 h, we can observe that 24 h supplementation period synchronized IVM and showed satisfactory results due uniformity of maturation among the three media. Usually, this uniformity provides more consistent embryo developmental rates.

That is interesting to note in the 24 h treatment, during transiction from 24 h until 32 h, all groups showed statistical differences. In addition, during IVM changes from 32 h until 36 h, also was observed difference in the 24 h treatment. In the media with 48 h treatment was not observed such uniformity. From 28 h, only NCSU23 showed higher nuclear maturation rate. From 32 h, only Whitten showed difference in comparison with other media.

With regard to hormonal supplementation is able to support high cAMP levels inside oocyte, from its withdrawal during second half of IVM, we can observe higher ICM rates in the 24 h treatment, when probably cAMP levels decreased and allowed GVBD and the resumption of meiosis.

Porcine follicular fluid (PFF) is an exsudate of blood plasma with different protein factor, glicoproteins ad steroids. It was added to three IVM media. In order, these factors can inhibit resume of meiosis in certain moments and to stimulate it in others. Specific functions of these factors does not well elucidate so far. We can infer that steroids are present in PFF can support cAMP in least levels sufficient to keep block of meiosis and to prevent GVBD. This PFF interference seems to occur during the first half of IVM. However, effect of PFF on IVM of porcine oocytes is controversial, depending on PFF collection.

Biochemical analysis of PFF shows differences with regard to estral cycle or follicular development [11]. Thus, PFF can act in different ways with its compounds and with other factors present in the IVM medium.

CONCLUSION

In conclusion, no effect of maturation media on meiotic progression was observed. However, the proportion of oocytes that reached metaphase II (MII) stage was higher when eCG + hCG were added for 24 h than 48 h mainly at the 44 h of maturation. In addition, no differences were observed in cleavage and blastocyst rates of the cultured embryos. However, embryos cultured in NCSU23 showed lower rates of hatching compared to other media. These results indicated no effect of maturation media on the fertilization and embryonic development even in the presence of cysteine, PFF and EGF, except for hatched embryos that these rates were lower in NCSU23.

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SOURCES AND MANUFACTURERS

All chemicals were purchased from Sigma-Aldrich, Oakville, ON, Canada.

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