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INFLUENCE OF HORMONES AND FOLLICULAR FLUID ON MATURATION OF PIG OOCYTES

INFLUÊNCIA DE HORMÔNIOS E LÍQUIDO FOLICULAR NA MATURAÇÃO DE OÓCITOS SUÍNOS

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SUMMARY

*To evaluate the effect of follicular fluid on **in vitro** maturation, pig oocytes were cultured in the presence of hormones where 10% of fetal calf serum (FCS), 10% of follicular fluid from large follicles (l-pFF), 10% of follicular fluid from medium follicles (m-pFF) or no supplement were added. When oocytes where matured in medium containing the hormones the addition of different supplements did not affect (P<0.05) nuclear maturation. However, changing the supplement altered the cytoplasmic maturation, with higher rate (P<0.05) observed in the l-pFF group. To determine the effect of the presence of hormone and/or supplement during maturation, the oocytes were cultured either in presence of TCM-199 alone, with hormones, with 10% l-pFF or with hormones and 10% l-pFF. The highest proportion of oocytes undergoing nuclear and cytoplasmic maturation was obtained when both hormones and follicular fluid were present. Cumulus expansion had a significant (P<0.05) effect on cytoplasmic maturation with the non-expanded groups showing a lower percentage of maturation in all groups. When the adequacy of gonadotropins levels were evaluated by adding higher or lower concentrations into the maturation medium neither beneficial nor detrimental effects were observed in either nuclear or cytoplasmic maturation. These results suggest that changes in the composition of the medium can alter the percentage of oocytes completing maturation. Follicular fluid combined with hormones was found to give better conditions for pig oocyte maturation **in vitro**.*

Key words: *follicular fluid, oocyte maturation, gonadotrophins, porcine*

RESUMO

O presente estudo avaliou o efeito da presença do líquido folicular e hormônios durante a maturação nuclear e citoplasmática em ovócitos suínos. Para avaliar o efeito do líquido folicular, os oócitos foram maturados in vitro em TCM-199 na presença de hormônios, em que o meio foi suplementado com 10% de soro fetal bovino (SFB), 10% de líquido folicular de folículos grandes (l-pFF), 10% de líquido folicular de folículos médios (m-pFF) ou nenhum suplemento. Quando os oócitos foram maturados no meio contendo hormônios, a adição de diferentes suplementos não afetou (P<0,05) a maturação nuclear. Entretanto, mudanças no suplemento provocaram uma alteração na maturação citoplasmática, sendo que as maiores taxas (P<0,05) foram observadas no grupo com l-pFF. Para determinar o efeito da presença de hormônios e/ou suplemento durante a maturação, os oócitos foram cultivados em TCM-199, TCM-199 com hormônios, TCM-199 com 10% l-pFF ou TCM-199 com hormônio e 10% l-pFF. A maior proporção de oócitos que sofreram maturação nuclear e citoplasmática ocorreu no grupo em que ambos, hormônios e líquido folicular, estavam presentes. A expansão do cumulus influenciou (P<0,05) na maturação citoplasmática, sendo que os oócitos sem expansão apresentaram menor taxa de maturação em todos os grupos. Quando diferentes concentrações de gonadotrofinas foram utilizadas durante a maturação, não foi observado nenhum efeito na maturação nuclear ou citoplasmática. Os resultados deste estudo sugerem que as mudanças na composição do meio podem afetar as taxas de maturação, sendo que o líquido folicular, combinado com hormônios, proporciona as melhores condições para maturação de oócitos suínos.

Palavras-chave: *líquido folicular, maturação, oócito, gonadotrofinas, suíno.*

INTRODUCTION

There is some suggestion that addition of blood serum as a supplement to the culture medium provides a superior environment for oocyte maturation. Bovine and porcine oocytes matured and fertilized *in vitro* were shown to be capable of developing to the blastocyst stage in serum-free medium (PINYOPUMMINYTR & BAVISTER,

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1991; ABEYDEERA ***et al.***, 1998). Nevertheless, superior fertilizability of bovine (LEIBFRIED-RUTLEDGE ***et al.***, 1986; ZUELK & BRACKETT, 1990) oocytes was obtained in medium containing serum. On the other hand, the use of follicular fluid to replace serum in order to increase the efficiency of maturation has been proposed, since the follicular fluid is composed of follicular secretions, which in vivo supports oocyte maturation. Results of some studies have shown a positive effect of follicular fluid on nuclear and cytoplasmic maturation (NAITO ***et al.***, 1989; RATH ***et al.***, 1995), while some investigators reported no beneficial influence of the follicular fluid in male pronucleus (MPN) formation (YOSHIDA ***et al.***, 1992).

Another factor that affects oocyte maturation ***in vitro*** is the presence of hormones, especially gonadotropins (LIU ***et al.***, 1997). Although the mechanism of action of LH and FSH during maturation is not very well understood, the addition of these hormones during maturation is a current procedure. The concentration of the gonadotropin added to the maturation medium varies from 0.5µg/mℓ to 10µg/mℓ (MATTIOLI ***et al.*** 1988; ZHENG & SIRARD, 1992; ROSE & BAVISTER, 1992); however, the circulating levels after the LH surge in the pig are not higher than 20ng/mℓ of FSH and 5.9ng/mℓ of LH (VAN DE WIEL ***et al.***, 1981). In addition, the concentration of these hormones in the follicular fluid has been shown in humans to be approximately 30% of the serum levels. Thus, the concentrations usually added to the maturation medium are much higher than those present in either the serum or the follicular fluid.

Therefore, the objectives of this study were to examine the effect of different supplements added to the medium on nuclear and cytoplasmic maturation and to determine the adequacy of the gonadotropin levels added during maturation *in vitro* of pig oocytes.

MATERIAL AND METHODS

The cumulus oocyte complexes (COC) were recovered by aspiration of 2mm to 5mm diameter follicles. After washing the COCs were transfered to culture medium and cultured for 42 hours at 39ºC in an atmosphere 5% CO_2 in air. The basic medium for maturation was TCM-199 supplemented with pyruvic acid (44mg/mℓ), 100IU/mℓ of penicillin, 50µg/mℓ of streptomycin, 25mM Hepes , 5µg/mℓ of LH , 2.5µg/mℓ of FSH, 0.5µg/mℓ estradiol-17-ß and 10% of fetal calf serum (FCS). Changes in the basic medium were made according to each aspect to be examined in oocyte maturation.

Porcine follicular fluid was withdrawn with a syringe from follicles of two sizes, medium-size (m-pFF) from 2mm to 5mm in diameter and large-size follicles (l-pFF) from 6mm to 8mm in diameter. After collection the follicular fluid was filtered and stored at -20ºC in 500µℓ aliquots. After culture the oocytes were fixed for 48 hours in acetic acid-alcohol (1:3) and then stained with 1% lacmoid in 45% glacial acetic acid. The maturational stage of each oocyte was determined by using phase contrast microscopy (400-2000x) with only the oocyte in metaphase II being considered nuclear matured. Oocytes having one female pronucleus (FPN) and at least one male pronucleus (MPN), after fertilization, were considered as cytoplasmic matured.

For ***in vitro*** fertilization, boar fresh semen samples were diluted in a 1:2 dilution with a commercial extender (Modema), and incubated for 24 hours at 16ºC. Before use the spermatozoa were washed twice with BSA-saline (10mg/100mℓ), and then incubated at 39ºC for 20-30 minutes, during which time the oocytes were processed.

After the maturation, oocytes from each treatment were washed and transfered to fertilization medium (YOSHIDA ***et al.***, 1990), which was composed of modified TCM-199 with 10% of FCS and 0.04g of caffeine/100mℓ, with pH of 7.4. The spermatozoa were diluted to make a final concentration of $5x10^5$ cells/mℓ and were incubated with the oocytes for 8 hours. After this time, the oocytes were transfered to a 50µℓ drop of BMOC-2 medium and cultured for an additional 12 hours. At the end of the incubation the oocytes were fixed and stained.

Experiment 1 evaluated the effect of follicular fluid during *in vitro* maturation. COCs were cultured in TCM-199 in the presence of hormones where 10% of FCS, 10% l-pFF, 10% m-pFF or no supplement were added. After maturation oocytes were fertilized *in vitro*, fixed and stained.

In experiment 2 the effect of the presence of hormone and/or supplement during maturation was determined. The oocytes were cultured either in presence of TCM-199 alone, TCM-199 with hormones (LH, FSH and estradiol), TCM-199 with 10% l-pFF (v/v) or TCM-199 with hormones and 10% l-pFF.

Experiment 3 was designed to determine whether cumulus expansion is an important factor for cytoplasmic maturation independent of the maturation conditions. The COCs were divided into three treatments: no supplement, 10% FCS and 10%

l-pFF. For IVF, the oocytes from each treatment were placed in separated dishes according with their cumulus appearance.

In the experiment 4 the adequacy of the levels of the LH and FSH used in our system was investigated. Oocytes were matured in TCM-199 supplemented with 10% l-pFF and estradiol in the absence of gonadotropin or in the presence of 0.25µg/mℓ and 0.5µg/mℓ, 2.5µg/mℓ and 5.0µg/mℓ or 25µg/mℓ and 50µg/mℓ of FSH and LH, respectively. After maturation and fertilization, the eggs were fixed and stained. Levels of LH were measured in the follicular fluid by a RIA method described previously (MATTERI ***et al.***, 1987).

All the data were analyzed by analysis of variance (SAS Institute Inc., Cary, NC 27512-8000, USA), for a randomized complete block design. When a significant (P<0.05) effect was detected, Least Significant Difference (LSD) test was performed to determine differences among treatment means

RESULTS AND DISCUSSION

When COCs were incubated in medium containing hormones the addition of different supplement did not affect (P<0.05) nuclear maturation (Table 1). Follicular fluid and serum have many common elements such as hormones, proteins and growth factors, and both have been shown as to be beneficial for maturation. Although the results of this study suggest that a protein supplement in the medium is not required for nuclear maturation, cytoplasmic maturation can be increased by adding different source of protein. The development to the pronuclei stage was significantly higher in l-pFF (P<0.05) than that observed in other treatment groups except for the m-pFF which did not differ from l-pFF. The various components of follicular fluid have been shown to change in different phases of follicular development (AINSWORTH ***et al.***, 1980). Therefore, the levels of maturation obtained with the l-pFF group suggest that maybe there is an effective substance/s that is present in l-pFF which is lacking or is present in lower concentration in m-pFF and serum. In fact, ROMERO-ARREDONDO & SEIDEL (1996) showed that follicular fluid obtained 20 hours after LH surge gave better results in the *in vitro* maturation than the 0 hour follicular fluid.

As shown in table 2, addition of supplement alone to the medium failed to cause significant stimulus in nuclear and cytoplasmic maturation, showing that follicular fluid in the absence of hormones is unable to support maturation. On the other hand, the addition of hormones to the medium, in the absence of pFF, did not improved the percentage of oocytes with MPN formation, suggesting that gonadotropins alone are also insufficient to provide suitable environment for maturation. A marked increase in nuclear and cytoplasmic maturation of pig oocytes was only obtained when both hormones and supplement were present in the medium. This is consistent with others reports which show that secretion of follicle cells and gonadotropins are needed to promote the better conditions for maturation of pig oocytes (RATH ***et al.***, 1995; LIU ***et al.***, 1997).

When the COCs were matured in different supplements and the results grouped according to cumulus expansion (Table 3), no COC underwent expansion in the absence of either the FCS or l-pFF. The expansion of cumulus cells has been reported to be induced *in vitro* by FSH (HILLENSJO & CHANNING, 1980) with the presence of serum required to retain the secreted hyaluronic acid within the cumulus cell complex (EPPIG, 1980). Therefore, the absence of some components in the medium explains the

Table 1 - Effect of different media supplements on *in vitro* maturation of pig oocytes.

Treatment	Oocytes Number	MII[1] Oocytes %± se	Oocytes penetrated %± se	Oocytes polyspermic %± se	Nºsperm/ Oocytes %± se	Oocytes with DSH[2] %± se	Oocytes with MPN[3] %± se
TCM-199+ H[4]	68	78.90[a] ± 0.23	72.30[a] ± 0.76	79.90[a] ± 0.19	3.38[a] ± 0.00	68.40[a] ± 0.44	25.50 [c] ± 0.14
TCM199+ H+FCS[5]	70	94.20[a] ± 0.54	93.70[a] ± 1.30	80.50[a] ± 0.52	3.72[a] ± 0.00	60.60[a] ± 0.14	42.10 [b] ± 0.23
TCM-199+ H+l-pFF[6]	65	92.20[a] ± 0.68	83.00[a] ± 1.40	81.30[a] ± 0.53	3.74[a] ± 0.00	72.90[a] ± 1.20	62.60[a] ± 0.20
TCM-199+ H+m-pFF[7]	68	84.10[a] ± 0.14	81.00[a] ± 0.15	80.00[a] ± 0.07	3.56[a] ± 0.00	61.10[a] ± 0.19	51.10[a,b] ± 0.11

[1] Oocytes at metaphase II after maturation period.
[2] Oocytes containing decondensed sperm head after *in vitro* fertilization.
[3] Oocytes containing one female pronucleus and at least one male pronucleus after *in vitro* fertilization.
[4] 5µg of LH/mℓ, 2.5µg of FSH/mℓ and 0.5µg of estradiol/mℓ.
[5] 10% of fetal calf serum.
[6] Follicular fluid collected from 6mm to 8mm diameter follicles of pig ovaries.
[7] Follicular fluid collected from 2mm to 5mm diameter follicles of pig ovaries.
[a,b,c] Within each column values with different superscripts are significantly different (P<0.05).

Table 2 - Effect of presence of hormones and/or follicular fluid on *in vitro* maturation of pig oocytes.

Treatment	Oocytes Number	MII[1] Oocytes %± se	Oocytes at GV[2] %± se	Oocytes penetrated %± se	Oocytes polyspermic %± se	Nºsperm/ Oocytes %± se	Oocytes with DSH[3] %± se	Oocytes with MPN[4] %± se
TCM-199	56	55.8[a] ± 0.32	33.3[a] ± 0.46	64.7[a] ± 0.76	80.1[a] ± 0.37	3.3[a] ± 0.30	34.9[b] ± 1.80	12.6[b] ± 1.60
TCM-199+FF[5]	60	52.2[a] ± 1.80	42.7[a] ± 1.60	60.7[a] ± 0.77	46.2[b] ± 0.86	2.9[a] ± 0.52	42.1[b] ± 0.14	12.2[b] ± 1.60
TCM-199+H[6]	58	71.6[a,b] ± 1.80	24.8[a] ± 0.17	67.4[a] ± 1.90	77.0[a] ± 0.27	3.0[a] ± 0.30	57.5[b,a] ± 0.82	25.8[a,b] ± 0.68
TCM-199+H+FF	45	91.1[b] ± 1.20	8.9[a] ± 1.20	87.4[a] ± 1.60	85.5[a] ± 0.37	4.2[a] ± 0.40	80.1[a] ± 0.78	63.1[a] ± 0.32

[1] Oocytes at metaphase II after maturation period.
[2] Oocytes at germinal vesicle stage after maturation period.
[3] Oocytes containing decondensed sperm head after *in vitro* fertilization.
[4] Oocytes containing one female pronucleus and at least one male pronucleus after *in vitro* fertilization.
[5] Follicular fluid collected from 6mm to 8 mm diameter follicle on pig ovaries.
[6] 5µg of LH/mℓ, 2.5µg of FSH/mℓ and 0.5µg of estradiol/mℓ.
[a,b] Within each column values with different superscripts are significantly different (P<0.05).

low percentage or the absence of expansion when no supplement was used.

Cumulus expansion was not an important aspect in obtaining nuclear maturation since similar (P>0.05) percentages of oocytes underwent nuclear maturation in COCs possessing either an expanded or non-expanded cumulus (Table 3). Therefore, the lower maturation rate observed in the non supplemented group could be more associated with the poor environment than with the cumulus expansion. However, the ability to form a MPN was markedly influenced (P<0.05) not only by the addition of a supplement to the maturation medium but also to the ability of the cumulus to undergo expansion during maturation (Table 3). Thus, expansion seems to be an important factor in the pig oocyte maturation according to the present study, because expansion of the cumulus was consistently associated with increase in cytoplasmic maturation, independent of the supplement present. This is supported by NAKAYANA ***et al.*** (1996), who reported that in the pig the synthesis of hyaluronic acid is dependent of the oocyte, therefore the expansion of the cumulus does indicate the presence of a healthy COC that can achieve cytoplasmic maturation.

The percentage of oocytes penetrated by sperm was lower (P<0.05) in the non supplemented group than in the FCS or l-pFF groups (Table 3). In addition, that group also showed no expansion of the cumulus, low decondensed sperm head and low MPN formation. Studies in the pig oocyte showed that only oocytes functionally connected to the cumulus cells throughout maturation could be successfully penetrated by sperm. In fact, GALEATI ***et al.*** (1991) established the effect of somatic cells on cortical granule distribution, and confirmed that premature loss of intercellular coupling can cause a premature migration of cortical granules. Therefore, the conditions of the non supplemented medium could cause a premature loss of contact between the oocyte and the cumulus cells, resulting in premature exocytosis of the cortical granules and subsequent change in the egg penetrability.

The follicular fluid used in this experiment contained almost undetectable levels of LH (0.33ng/mℓ). The addition of 2.5µg/mℓ of FSH and 5.0µg/mℓ of LH used in our system stimulated a significant increase in the nuclear and cytoplasmic (P<0.05) maturation (Tables 1 and 2). When the adequacy of those levels were evaluated, by adding higher or lower concentrations of the gonadotropins into the maturation medium, neither beneficial nor detrimental effects were observed in nuclear or cytoplasmic maturation (Table 4). The site of action and mechanism of FSH and LH in the COC is unknown although their beneficial effects are well defined. One possibility is that gonadotropins stimulate some substance on the cumulus and/or granulosa cells which would act in the oocyte. This possibility is supported by the results of studies on localization of LH receptors in rat ovaries (BUROVSKY ***et al.***, 1993), which showed the presence of LH receptors in granulosa cells surrounding the oocytes of healthy preantral follicles. In fact, MATTIOLI & BARBONI (1998) suggested that the somatic cells read the LH signal and then they elaborate a message which is send to the oocyte inducing maturation. The concentrations of LH and FSH used in this experiment seem to be unnecessary, since much lower concentrations can be used in the maturation medium without losing the effectivess in improving maturation.

Table 3 - Characteristics of pig oocytes following maturation in various media, categorized according to expansion of the cumulus complex.

Treatment	Oocytes (Nº)	MII[1] oocytes %±se	Oocytes Penetrated %±se	Polyspermic Oocytes %±se	Nº sperm/ Polyspermic oocyte	Oocytes with DSH[2] %±se	Oocytes with MPN[3] %±se
FCS							
Expanded[4]	75	89,30[a,b] ± 1.10	72.60[a,c] ± 1.40	41.40[a] ± 0.94	2.80[a] ± 0.50	64.50[b,c] ± 1.10	33.70[a] ± 0.82
Not expanded[5]	29	81.60[a,b] ± 0.97	62.30[a,c] ± 2.90	31.70[a] ± 5.50	3.80[a] ± 0.01	99.30[a] ± 0.59	3.20[b] ± 1.30
L-pFF							
Expanded	74	89.50[a,b] ± 0.46	84.10[a] ± 0.95	33.20[a] ± 2.00	2.50[a] ± 0.00	68.40[b,c] ± 1.70	47.30[a] ± 0.46
Not Expanded	25	96.40[a] ± 1.60	80.40[a] ± 3.70	37.40[a] ± 9.20	3.60[a] ± 0.05	96.90[a,c] ± 3.10	0.86[b] ± 0.86
No supplement							
Expanded	0	–	–	–	–	–	–
Not Expanded	82	65.60[b] ± 0.17	43.90[b] ± 1.10	22.40[a] ± 0.68	2.50[a] ± 0.001	40.30[b] ± 4.80	3.90[b] ± 0.99

[1] Oocytes in metaphase II after 48 hours of maturation.
[2] DSH- Decondensed sperm head.
[3] MNP- Male pronucleus formation.
[4] Oocytes with expanded cumulus cells after maturation period.
[5] Oocytes with non expanded cumulus cells after maturation period.
[a,b,c] Within each column values with different superscripts are significantly different (P<0.05).

CONCLUSION

The results of this study indicated that neither supplement nor hormone alone are capable of supporting pig oocyte ***in vitro*** maturation; cytoplasmic maturation rates can be increased by supplementing with follicular fluid, especially that obtained from large follicles; lower concentrations of gonadotropins can be used during maturation with no detrimental effect in maturation.

Table 4 - Effect of various levels of gonadotropins on maturation of pig oocytes.

Concentration[1] of LH (µg/mℓ)	FSH (µg/mℓ)	Oocytes (Nº)	MII[2] Oocytes %±se	Oocytes Penetrated %±se	Oocytes Polyspermic %±se	Sperm/ Oocytes %±se	Oocytes With DSH[3] %±se	Oocytes With MPN[4] %±se
0.00	0.00	60	46.60[a] ±0.99	60.50[a] ±1.50	64.90[a] ±3.60	2.95[a] ±0.34	45.70[a] ±1.90	4.50[a] ±1.70
0.50	0.25	65	90.40 [b] ±1.20	86.30[a] ±0.44	73.50[a] ±1.70	3.25[a] ±0.61	58.10[a] ±3.30	59.10 [b] ±0.21
5.00	2.50	61	94.80 [b] ±0.60	81.90[a] ±2.00	77.80[a] ±1.40	3.42[a] ±0.42	73.20[a] ±0.88	56.90 [b] ±0.08
50.00	25.00	67	95.10 [b] ±0.62	84.60[a] ±0.51	72.70[a] ±1.60	3.12[a] ±0.39	73.10[a] ±0.86	58.80 [b] ±0.19

[1] Concentration of gonadotropins added to a modified TCM-199 supplemented with 10% of follicular fluid aspirated from 6-8mm of diameter follicles.
[2] Oocytes at metaphase II stage after *in vitro* maturation.
[3] Oocytes with decondensed sperm head after *in vitro* fertilization.
[4] Oocytes with one female pronucleus and at least one male pronucleus after *in vitro* fertilization.
[a,b] Within each column values with different superscripts are significantly different (P<0.05).

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