

INFLUENCE OF DIFFERENT FOLLICULAR FRACTIONS ON *IN VITRO* MATURATION OF IMMATURE BOVINE OOCYTES*

INFLUENCIA DE DIFERENTES FRACCIONES FOLICULARES SOBRE LA MADURACIÓN *IN VITRO* DE OVOCITOS INMADUROS DE BOVINO*

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ADDITIONAL KEYWORDS

Granulosa cells.

PALABRAS CLAVE ADICIONALES

Células de la granulosa.

SUMMARY

Three experiments were carried out to investigate whether cumulus cells, Bovine Follicular Fluid (BFF) and granulosa cells, when added as supplements to the maturation medium, support the *in vitro* maturation of immature bovine oocytes. In the first experiment, the maturation medium was supplemented with three different concentrations of the BFF (10, 20 and 50 p.100, respectively). In the second experiment, the effect of three different concentrations of the granulosa cells (1, 10 and 100x10⁶ cells/ml, respectively) on *in vitro* oocyte maturation percentages were evaluated. In the third experiment, the effect of the presence or absence of cumulus cells on the *in vitro* oocyte maturation rates was compared.

Maturation rates at the metaphase II stage, following supplementation with 20 p.100 BFF (60 p.100) were significantly higher ($p < 0.05$) than those obtained in control group (45 p.100). Significant differences between the 10 p.100 BFF, 20 p.100 BFF and 50 p.100 BFF treatments, were not observed. The results obtained from the 1x10⁶ granulosa cells/

ml and the control treatments (90 p.100 and 85 p.100, respectively) were significantly higher ($p < 0.001$) than when 10 and 100x10⁶ cells/ml (66 p.100 and 47 p.100, respectively) were added. Significant differences in the presence (78 p.100) or absence (62 p.100) of cumulus cells ($p < 0.05$) on *in vitro* oocyte maturation rates were found. The present results indicate that the presence of cumulus cells surrounding immature oocytes, the addition of low granulosa cells concentrations and BFF added to the maturation medium may be required to induce both nuclear and cytoplasmic maturation of immature bovine oocytes *in vitro*.

RESUMEN

Se llevaron a cabo tres experimentos con el objetivo de investigar si las células del cúmulo, fluido folicular bovino (FFB) y las células de la granulosa, cuando son añadidos como suplemento al medio de maduración, soportan la maduración *in vitro* de ovocitos inmaduros de bovino. En el primer experimento, el medio de maduración fue suplementado con tres diferentes concentraciones de FFB (10, 20

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y 50 p.100, respectivamente). En el segundo experimento, se evaluó el efecto de tres concentraciones diferentes de células de la granulosa (1, 10 y 100×10^6 células/ml, respectivamente), sobre los porcentajes de maduración ovocitaria *in vitro*. En el tercer experimento, se comparó el efecto de la presencia o ausencia de células del cúmulo sobre los índices de maduración ovocitaria *in vitro*.

Los índices de maduración en el estadio de metafase II, siguientes a la suplementación con un 20 p.100 FFB (60 p.100) fueron significativamente superiores ($p < 0.05$) a los obtenidos en el grupo control (45 p.100). No se encontraron diferencias significativas entre los tratamientos de 10 p.100 FFB, 20 p.100 FFB y 50 p.100 FFB. En el segundo experimento, los resultados obtenidos en los tratamientos de 1×10^6 células/ml y control (90 p.100 y 85 p.100, respectivamente) fueron significativamente superiores ($p < 0.001$) a los obtenidos con 10 y 100×10^6 células/ml (66 p.100 y 47 p.100, respectivamente). En el tercer experimento, se encontraron diferencias significativas entre la presencia (78 p.100) o la ausencia (62 p.100) de células del cúmulo, sobre la maduración ovocitaria *in vitro*. Los resultados indican que la presencia de células del cúmulo alrededor de los ovocitos inmaduros, la adición de bajas concentraciones de células de la granulosa y la adición de FFB al medio de maduración pueden ser requeridas para inducir tanto la maduración nuclear como citoplasmática *in vitro* de ovocitos inmaduros de bovino.

INTRODUCTION

Meiotic maturation occurs spontaneously when bovine oocytes are removed from their follicles and cultured *in vitro*. This natural phenomenon in mammals has greatly facilitated the use of *in vitro* matured oocytes for fertilization and embryo production. Bovine oocytes from small antral (immature) follicles can be matured *in vitro* up to the stage where they can be fertilized but their average subsequent developmental

capacity is limited. It is clear that normal cytoplasmic maturation must occur *in vitro* to produce a good embryo but, is the cytoplasm of all oocytes ready to respond to our maturation conditions? if we suppose that a maturing follicle influences the oocyte's ability to support further development, it is essential to understand this process and simulate this effect *in vitro*.

In mammals, nuclear maturation, normally induced *in vivo* by the LH surge, occurs spontaneously *in vitro* when oocytes are removed from follicles and cultured in simple medium (Pincus and Enzmann, 1935). Two major events are involved in this process. First, the cumulus-oocyte complex (COC) is removed from the influence of the follicular micro-environment (follicular fluid). Second, physical contact with mural granulosa cells is ruptured, terminating intercellular communication via the gap junctions. This chemico-physical stimulation of the oocyte causes condensation of the chromatin and the breakdown of the nuclear membrane (germinal vesicle), leading to metaphase-II and a second artificial arrest in the cycle (Edwards, 1965). To explain this spontaneous phenomenon, it was postulated that the follicle produces an inhibitory factor preventing meiotic resumption, since in fetal gonads of females, meiotic arrest is associated with the presence of somatic cells forming the primordial follicle (Wassarman, 1988).

The objective of this experiment was to investigate the influence of three follicular components, such as cumulus cells, granulosa cells and follicular fluid on *in vitro* maturation of immature bovine oocytes. These components are known for being involved in the meiotic maturation process.

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MATERIALS AND METHODS

COLLECTION AND MATURATION OF BOVINE FOLLICULAR OOCYTES

Ovaries were collected at a slaughter, placed into physiological saline (0.9 p.100, w/v, NaCl) with antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) maintained at 30 to 37°C, and transported to the laboratory within 1 to 2 h of slaughter. Cumulus-oocyte complexes were aspirated from small (1-5 mm diameter) antral follicles with an 18 gauge 1" needle attached to a 5 ml disposable syringe containing modified phosphate buffered saline (PBS) supplemented with 5 p.100 (v/v) heat-inactivated fetal calf serum (FCS; Sigma) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). Oocytes were washed three times with Hank's balanced salt solution supplemented with antibiotics, and 5 p.100 (v/v) FCS (washing medium). The basic maturation medium was TCM-199 with Earle's salts (Sigma) supplemented with 2,5 mM HEPES (Sigma) and antibiotic-antimycotic solution (Sigma). Oocytes with an intact cumulus cell were cultured in 1 ml TCM-199 maturation medium and incubated at 39°C, 5 p.100 CO₂ in air for 24 h. The follicular components were added as described below.

PREPARATION OF GRANULOSA CELLS AND BFF

The granulosa cells were collected from follicles 1 to 5 mm in diameter. The follicular fluid was aspirated with a 10 ml syringe and an 18-g 1" needle. After removal of the oocytes, the remaining follicular fluid was centrifugated at 250 g for 10 min. The pellet containing granulosa cells was washed once in

Hank's balanced salt solution and twice in maturation medium by centrifugation at 250 g for 10 min. Subsequently, the pellet was re-suspended in maturation medium. The concentration of granulosa cells used was 1x10⁶ cells/ml to 100x10⁶ cells/ml in co-culture with oocytes. The supernatant fluid was filtered (0.2 µm pore size), supplemented with antibiotic-antimycotic solution (Sigma) and stored frozen at -20°C until required for the culture of oocytes.

EXPERIMENTAL DESIGN

Experiment 1

Three concentrations of BFF were added to the maturation medium of immature bovine oocytes, the oocytes were matured for 24 h according to the following scheme: (1) the oocytes were cultivated in the culture medium TCM-199, supplemented with 10 p.100 BFF; (2) the oocytes were matured in the basic maturation medium supplemented with 20 p.100 BFF; (3) the oocytes were matured for 24 h in the maturation medium enriched with 50 p.100 BFF; (4) the oocytes were cultivated in the culture medium TCM-199 alone without BFF supplement (control group).

Experiment 2

Three concentrations of granulosa cells were added to the maturation medium of preovulatory bovine oocytes, the oocytes were matured for 24 h according to the following scheme: (1) the oocytes were co-cultivated in the culture medium with 1x10⁶ granulosa cells/ml; (2) the oocytes were co-cultivated in the basic maturation medium with 10x10⁶ granulosa cells/ml; (3) the oocytes were co-cultivated with 100x10⁶ granulosa

Table I. Percentages of development stage on *in vitro* maturation of bovine oocytes in TCM-199 medium supplemented with different concentrations of BFF after 24 h of culture. (Porcentajes en los diferentes estadios de desarrollo de la maduración *in vitro* de ovocitos bovinos cultivados en el medio TCM-199 suplementado con diferentes concentraciones de FFB después de 24 horas de cultivo).

BFF (p.100)	Trials (N)	Oocytes (N)	Nuclear stage (p.100)		
			GV	M-I	M-II
10	3	60	18(30)	13(21)	29(49) ^{ab}
20	3	100	36(36)	4(4)	60(60) ^{ac}
50	3	60	15(25)	12(20)	33(55) ^{ab}
Control	3	80	34(43)	10(12)	36(45) ^{bd}

GV: germinal vesicle; M-I: metaphase-I; M-II: metaphase-II.

^{a,b,c,d}Values with different superscripts within each column are statistically different; ^{c,d}values differ significantly ($p < 0.05$).

cells/ml; (4) the oocytes were not co-cultivated in the basic maturation medium with granulosa cells and was used as control group.

Experiment 3

Oocytes were matured for 24 h according to the following scheme; (1) the cumulus-enclosed oocytes were cultivated in the basic maturation medium; (2) the cumulus-denuded oocytes were incubated in the maturation medium.

CHROMOSOME PREPARATION OF THE OOCYTES

At the end of the culture period for maturation, the oocytes were transferred to 3 ml conical tubes and vortex-agitated for 2 min in trisodium citrate (0.88 p.100) and trypsin (0.02 p.100) hypotonic solution. After a slight agitation to remove the COC, denuded oocytes were transferred to a culture plate containing 2 ml of the same hypotonic solution but without trypsin for 45-60 min. The

oocytes were fixed in an initial fixing solution of 1:1 methanol:acetic acid followed by a second solution of 3:1 methanol:acetic acid for 24 h. Finally, the oocytes were mounted on slides, stained with 5 p.100 Giemsa and examined with the light microscope at 400 and 1500 x magnification for evaluation of maturation.

CRITERIA FOR MATURATION AND STATISTICAL ANALYSIS

Oocytes were morphologically evaluated for stage of maturation after culture. The meiotic progress of oocytes was classified as follows: (1) germinal vesicle stage, an intact nuclear membrane with the chromatin meiotically inactive; (2) Metaphase I, the nuclear membrane broken and a chromatin pattern characteristic of an oocyte resuming meiosis; (3) Metaphase II, a polar body present within the perivitelline space with maternal chromatin complement identified in the oocyte and (4) degeneration, oocyte showing obvious

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degenerative signs such as vacuolated or fragmented cytoplasm or scattered chromatin complement. Data were statistically analysed by Chi-Square test (S.A.S Institute Inc., 1982).

RESULTS

EXPERIMENT 1

Results of maturation of the oocytes matured in TCM-199 medium and supplemented with different concentrations of BFF are shown in **table I**. When the immature oocytes were matured in the presence of 20 p.100 BFF (60 p.100), the percentage reaching metaphase II stage was significantly higher ($p < 0.05$) than to that found in the control group. However, significant differences between the 10 p.100, 20 p.100 and 50 p.100 BFF treatments were not detected.

EXPERIMENT 2

The effects of the addition of granulosa cells to the maturation medium on oocyte

maturation rates are shown in **table II**. The oocytes matured in the TCM-199 medium with a supplement of 100×10^6 granulosa cells/ml (47 p.100) showed lower maturation percentages ($p < 0.001$ and $p < 0.05$, respectively) than the oocytes matured in the presence of 1×10^6 cells/ml (90 p.100), control group (85 p.100) and 10×10^6 cells/ml (66 p.100). Thus, when the oocytes were matured in the presence of 10×10^6 cells/ml (66 p.100) the percentage reaching metaphase II stage was significantly lower ($p < 0.01$) than to that found in the group control (85 p.100). However, significant differences between the 1×10^6 cells/ml and control treatments were not observed.

EXPERIMENT 3

The results of the maturation rate of the oocytes matured in the presence or absence of cumulus cells are shown in **table III**. The cumulus-enclosed oocytes (78 p.100) had higher maturation rates ($p < 0.05$) than those found in the denuded oocytes group (62 p.100).

Table II. Effect of granulosa cells added to culture medium for in vitro maturation of bovine oocytes after 24 h of culture. (Efecto de las células de la granulosa añadidas al medio de cultivo durante la maduración *in vitro* de ovocitos bovinos después de 24 horas de cultivo).

Granulosa cells ($\times 10^6$ /ML)	Trials (N)	Oocytes (N)	Nuclear stage (p.100)		
			GV	M-I	M-II
1.0	3	80	4(5)	4(5)	72(90) ^{af}
10.0	3	75	19(25)	7(9)	50(66) ^{bd}
100.0	3	75	36(48)	4(5)	35(47) ^c
Control	3	85	5(6)	8(9)	72(85) ^{ae}

GV: germinal vesicle; M-I: metaphase-I; M-II: metaphase-II. ^{a-f}Values with different superscripts within each column are statistically different. ^{b-c}values differ significantly ($p < 0.05$). ^{d-e}values differ significantly ($p < 0.01$). ^{f-b}; ^{f-c} and ^{a-c}values differ significantly ($p < 0.001$).

Table III. *In vitro* maturation rates of cumulus-enclosed and denuded oocytes cultured in TCM-199 medium after 24 h of culture. (Índices de maduración *in vitro* de ovocitos cubiertos con células del cúmulo y desnudos cultivados en el medio TCM-199 después de 24 horas de cultivo).

Oocyte type	Trials (N)	Oocytes (N)	Nuclear stage (p.100)		
			GV	M-I	M-II
Enclosed	3	70	9(14)	6(8)	55(78) ^a
Denuded	3	65	15(23)	10(15)	40(62) ^b

GV: germinal vesicle; M-I: metaphase-I; M-II: metaphase-II.

^{a,b}Values with different superscripts within each column are statistically different. ^{a,b}values differ significantly ($p < 0.05$).

DISCUSSION

Many workers have investigated whether hormones (Critser *et al.*, 1986; Fukui *et al.*, 1991), serum (Goto *et al.*, 1988), granulosa cells (Zheng and Sirard, 1992), cumulus cells surrounding immature oocytes (Sirard *et al.*, 1988) and protein supplements (Sirard *et al.*, 1992) are necessary for *in vitro* maturation.

The present study indicates that the maturation of cumulus-enclosed oocytes was superior to that of denuded oocytes. It has been reported that cumulus cells surrounding the oocytes (COC) (DeFelici and Siracusa, 1982; Katska *et al.*, 1989) and serum (Downs *et al.*, 1986) protect the zona pellucida against hardening. Thus, Mochizuki *et al.* (1991) found that denuded oocytes had low fertilizability due to the hardening of the zona pellucida and incomplete cytoplasmic maturation due to the disconnection of junctional complexes. It is known that the nutrients required for oocyte maturation are transported into the ooplasm via the

junction gap between the ooplasm and cumulus cells (Sirard *et al.*, 1992; Dekel *et al.*, 1984). Fukui and Sakuma, (1980) and Shioya *et al.* (1988) have reported that follicle cells, especially the cumulus cells surrounding immature oocytes, play a central role in developmental competence in inducing not only nuclear maturation but also cytoplasmic maturation. Goto *et al.* (1988), Ball *et al.* (1984) and Sanbuissho and Threlfall (1990) indicate that mucinate expansion of cumulus cells after *in vitro* maturation is a sign of both nuclear and cytoplasmic maturation.

Several workers (Fukui *et al.*, 1991; Akufo *et al.*, 1988) have also used granulosa cells to induce nuclear and cytoplasmic maturation of bovine follicular oocytes, and indicate that these cells enhance the fertilizability and subsequent developmental competence of the oocytes. However, it is not clear that granulosa cells concentration is beneficial or detrimental on *in vitro* oocyte maturation. In our study, three different concentrations of granulosa cells (1, 10

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and 100×10^6 cells/ml) were used. The group of oocytes co-cultured in maturation medium with 1×10^6 cells/ml resulted in higher normal maturation rate than in groups to which no cells were added or to which 10 or 100×10^6 cells/ml were added. The present results confirmed those previous studies (Sirard *et al.*, 1992; Lu *et al.*, 1988), but a higher concentration of granulosa cells supplemented with culture medium failed to improve maturation (10 to 100×10^6 cells/ml) of the oocytes. This negative effect may have been due to energy deficiency required for oocyte maturation, because increased numbers of granulosa cells require more nutrients and respiratory metabolism in the culture medium.

There are few published reports on the effects of follicular fluid on bovine oocytes in the literature. However, a previous study (Sirard and First, 1988) reported that BFF significantly reduced the proportion of oocytes resuming meiosis at 6 h of maturation, but after 21 h of culture BFF had no more effect on maturation rates compared to control. In our study, when maturation medium was supplemented with BFF the maturation rates were higher than to those obtained in control treatment to which BFF was not added. The best results were obtained when the culture medium was enriched with 20 p.100 BFF. We suggest that BFF supplement might to contain important levels of protein and growth factors that improve the oocyte maturation rates. However, the maturation rates obtained in BFF-supplemented medium are lower than to those obtained with other protein supplements widely used in the maturation medium (Sanbuissho and Threlfall, 1990; Nakanishi *et al.*, 1990). It might be due

that BFF contains nucleotic that inhibit resumption of meiosis in bovine oocytes. Sato and Koide, (1988) isolated a small peptide (< 10KD) from bovine follicular fluid capable of inhibiting meiotic resumption of mouse oocytes in culture. Earlier, Tsafriri *et al.* (1976) found a small proteolytic-sensitive molecule in the porcine follicular fluid that could be active in preventing mouse meiotic resumption, as shown by Downs and Eppig (1984). Sirard *et al.* (1992) reported that in BFF might to exists an OMI peptide that is an inhibiting factor of the maturation. Besides this peptide, other components, such as hipoxantine and cAMP factor that increase in inhibition of maturation.

In conclusion, much evidence supports the hypothesis that multiple follicular factors combine to create the environment in which the germinal vesicle is maintained. We suggest that mucinate expansion of cumulus cells may indicate oocyte maturation, and cumulus cells of immature oocytes and granulosa cells (1×10^6 cells/ml) added to culture medium are essential for oocyte nuclear and cytoplasmic maturation. Finally, the bovine follicular fluid might to contain active molecules that can be inhibitory of meiotic resumption of preovulatory bovine oocytes.

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REFERENCES

- Akufo, E., D.A. Phelps, F.D. Tibbitts and W.D. Foote. 1988. Influence of follicular components on oocyte meiosis *in vitro*. *Theriogenology*, 30: 643-648.
- Ball, G.D., M.L. Leibfried, R.L. Ax and N.L. First. 1984. Maturation and fertilization of bovine oocytes *in vitro*. *J. Dairy Sci.*, 67: 2775-2785.
- Critser, E.S., M.L. Leibfried-Rutledge, W.H. Eyestone, D.L. Northey and N.L. First. 1986. Aquisition of developmental competence during maturation *in vitro*. *Theriogenology*, 25: 150 abstr.
- DeFelici, M. and G. Siracusa. 1982. Spontaneous hardening of the zona pellucida of mouse oocytes during *in vitro* culture. *Gamete Res.*, 6: 107-113.
- Dekel, N., E. Aberdam and I. Sherizly. 1984. Spontaneous maturation *in vitro* of cumulus enclosed rat oocytes is inhibited by forskolin. *Biol. Reprod.*, 31: 344-350.
- Downs, S.M. and J.J. Eppig. 1984. Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. *Endocrinology*, 114: 418-427.
- Downs, S.M., A.C. Schroeder and J.J. Eppig. 1986. Serum maintains the fertilizability of mouse oocytes matured *in vitro* by preventing hardening of the zona pellucida. *Gamete Res.*, 15: 115-122.
- Edwards, R. 1965. Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*, 208: 349-351.
- Fukui, Y. and Y. Sakuma. 1980. Maturation of bovine oocyte cultures *in vitro*: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. *Biol. Reprod.*, 22: 669-673.
- Fukui, Y., L.T. McGowan, R.W. James, P.A. Pugh and H.R. Tervit. 1991. Factors affecting the *in vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. *J. Reprod. Fertil.*, 92: 125-131.
- Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa. 1988. Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. *J. Reprod. Fertil.*, 83: 735-758.
- Katska, L., P. Kauffold, Z. Smorag, U. Duschinski, H. Torner and W. Kanitz, W. 1989. Influence of hardening of the zona pellucida on *in vitro* fertilization of bovine oocytes. *Theriogenology*, 32: 767-777.
- Lu, K.H., I. Gordon, H.B. Chen, M. Gallagher and H. McGovern. 1988. Birth of twins after transfer of cattle embryos produced by *in vitro* techniques. *Vet. Rec.* 122: 539-540.
- Mochizuki, H., Y. Fukui and H. Ono. 1991. Effect of the number of granulosa cells added to culture medium for *in vitro* maturation, fertilization and development of bovine oocytes. *Theriogenology* 36: 973-986.
- Nakanishi, Y., Y. Uto, K. Goto and K. Ogawa. 1990. The effect of different media and serum supplements upon porcine oocyte maturation. *Theriogenology*, 33: 291.
- Pincus, G. and E.V. Enzmann. 1935. The comparative behavior of mammalian eggs *in vitro* and *in vivo*. *J. Exp. Med.*, 62: 665-675.
- S.A.S institute Inc. SAS user's guide: Basic 1982.
- Sanbuissho, A., Threlfall, W.R. 1990. The influence of serum and gonadotropins on *in vitro* maturation and fertilization of bovine oocytes. *Theriogenology*, 34: 341-348.
- Sato, E. and S.S. Koide. 1988. A factor from bovine

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- granulosa cells preventing oocyte maturation. *Differentiation*, 39: 229-234.
- Shioya, Y., M. Kuwayama, M. Fukuishima, S. Iwasaki and A. Hanada. 1988. *In vitro* fertilization and cleavage capacity of bovine follicular oocytes classified by cumulus cells and matured *in vitro*. *Theriogenology*, 30: 489-496.
- Sirard, M.A. and N.L. First. 1988. *In vitro* inhibition of oocyte nuclear maturation in the bovine. *Biol. Reprod.*, 39: 229-234.
- Sirard, M.A., J.J. Parrish, C.B. Ware, M.L. Leibfried-Rutledge and N.L. First. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. *Biol. Reprod.*, 39: 546-552.
- Sirard, M.A., K. Coenen and S. Bilodeau. 1992. Effect of fresh or cultured follicular fractions on meiotic resumption in bovine oocytes. *Theriogenology*, 37: 39-57.
- Tsafri, A., S.H. Pomerantz and C.P. Channing. 1976. Inhibition of oocyte maturation by porcine follicular fluid: partial characterization of the inhibitor. *Biol. Reprod.* 14: 511-516.
- Wassarman, P.M. 1988. The mammalian ovum. In: *The Physiology of Reproduction*. E. Knobil and J. Neill (eds.) Raven Press, New York: pp. 69-102 (1988).
- Zheng, Y.S. and M.A. Sirard. 1992. The effect of sera, bovine serum albumin and follicular cells on *in vitro* maturation and fertilization of porcine oocytes. *Theriogenology*, 37: 779-790.

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