

# INFLUENCE OF THE *IN VITRO* BOVINE OOCYTES MATURATION CONDITIONS ON FERTILIZATION RATES

## INFLUENCIA DE LAS CONDICIONES DE MADURACIÓN *IN VITRO* SOBRE LOS ÍNDICES DE FERTILIZACIÓN DE OVOCITOS DE BOVINO

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### ADDITIONAL KEYWORDS

Bovine. Oocyte. *In vitro* fertilization. *In vitro* maturation.

### PALABRAS CLAVE ADICIONALES

Bovino. Maduración *in vitro*. Fecundación *in vitro*. Ovocito.

### SUMMARY

The influence of maturation medium, serum and hormonal supplements, type of bovine oocyte, as well as the co-culture with granulosa cells for *in vitro* bovine oocytes maturation on the *in vitro* fertilization rate were investigated. Immature oocytes with cumulus cells were cultured with two different maturation media (M-199 and DMEM; Experiment 1). In Experiment 2, *in vitro* maturation was performed with two serum supplements and one hormonal supplement (FCS, ECS and PMSG+hCG). In Experiment 3, *in vitro* maturation was performed with two different bovine oocyte types (CEO and CDO). In Experiment 4, *in vitro* maturation was performed with or without the addition of granulosa cells. After *in vitro* oocyte maturation, oocytes were fertilized using frozen-thawed semen which was previously capacitated with bovine serum albumin and heparin for 15 minutes.

The M-199 showed a fertilization rate higher (55 percent;  $p < 0.01$ ) than those obtained in DMEM (39 percent; Experiment 1). In Experiment 2, the serum and hormonal supplements showed *in vitro* fertilization rates higher (FCS:55 percent, ECS:57 percent, PMSG + hCG: 60 percent;  $p < 0.001$ ) than that in control

group (31 percent). In Experiment 3, *in vitro* fertilization rate of cumulus-denuded oocytes (30 percent) was lower ( $p < 0.001$ ) than those of cumulus-enclosed oocytes (60 percent). In Experiment 4, oocytes co-cultured with granulosa cells had higher rates of fertilization (70 percent;  $p < 0.05$ ) than oocytes matured without co-cultured with granulosa cells (55 percent).

The results indicate that the use of M-199 medium, the addition of serum or hormonal supplements, the oocyte type and the co-culture with granulosa cells during *in vitro* maturation influenced the fertilizability of *in vitro* matured oocytes.

### RESUMEN

Se investigó la influencia del medio de maduración, los suplementos séricos y hormonales, tipo de ovocito bovino, así como el co-cultivo con células de la granulosa durante la maduración oocitaria *in vitro* sobre los índices de fertilización *in vitro* de ovocitos bovinos.

Ovocitos inmaduros con células del cúmulo se cultivados en dos medios de maduración diferentes (M-199 y DMEM; experimento 1). En el experimento

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2, la maduración *in vitro* se realizó utilizando dos suplementos séricos y uno hormonal (FCS, ECS y PMSG+hCG). En el experimento 3, la maduración se realizó utilizando dos tipos de ovocitos diferentes (CEO y CDO). Y en el experimento 4, la maduración *in vitro* se llevó a cabo con o sin la adición de células de la granulosa. Después de la maduración de los ovocitos se procedió a su fecundación *in vitro* utilizando semen descongelado que fue capacitado previamente con suero albuminoso de bovino y heparina durante 15 minutos.

El medio M-199 mostró un índice de fecundación superior (55 p.100;  $p < 0,01$ ) que aquel obtenido por el medio DMEM (39 p.100; experimento 1). En el experimento 2, los suplementos séricos y hormonales mostraron un índice de fecundación superior (FCS:55 p.100, ECS:57 p.100, PMSG+hCG:60 p.100;  $p < 0,001$ ) al del grupo control (31 p.100). En el experimento 3, el índice de fecundación *in vitro* de los ovocitos desnudos fue más bajo (30 p.100;  $p < 0,001$ ) que aquellos obtenidos por los ovocitos cubiertos por células del cúmulo (60 p.100). En el experimento 4, ovocitos co-cultivados con células de la granulosa tuvieron índices de fecundación superiores (70 p.100;  $p < 0,05$ ) que los ovocitos madurados sin co-cultivarse con células de la granulosa (55 p.100).

Los resultados indican que el uso del medio M-199, la adición de suero o suplementos hormonales, el tipo de ovocito y el co-cultivo con células de la granulosa durante la maduración *in vitro* influyen en la capacidad de fecundación de ovocitos madurados *in vitro*.

## INTRODUCTION

It is well established that the culture conditions employed for *in vitro* maturation of mammalian oocytes can significantly influence *in vitro* fertilization rates and subsequent embryonic development (Brackett *et al.* 1989; Zuelke and Brackett, 1990). A recent comparison of several commercially available complex chemically defined cell culture

media revealed that *in vitro* maturation in Ham's F-12 resulted in significantly reduced fertilization of bovine oocytes compared with *in vitro* maturation in either TCM-199 or MEM (Rose and Bavister, 1992).

Immature bovine oocytes recovered from ovarian follicles are capable of resuming meiosis in the absence of serum (Suss *et al.*, 1988). However, fertilization and embryonic development of *in vitro* matured bovine oocytes was superior after *in vitro* maturation in gonadotropin hormones and serum-containing medium (Leibfried-Rutledge *et al.*, 1986; Zuelke and Brackett, 1992). Sirard *et al.* (1988), Shioya *et al.* (1988), and Fukui (1990) have demonstrated 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. Mochizuki *et al.* (1991) reported that the addition of granulosa cells to the culture medium improved *in vitro* fertilization and developmental capacity of bovine follicular oocytes in cumulus cells.

The objective of this study was to determine the influence of different maturation conditions on *in vitro* fertilization rates of *in vitro* matured bovine oocytes.

## MATERIALS AND METHODS

### PREPARATION OF ECS SUPPLEMENT

Blood was collected by yugular venepuncture from cows in the first 8 to 20 h of estrous and centrifuged at 500 g for 10 min. The serum was inactivated by heating at 56°C for 30 min and stored at -20°C until used for oocyte culture.

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### PREPARATION OF GRANULOSA CELLS

Granulosa cells were collected from antral follicles of 10 to 15 mm diameter after dissection and then washed (500 g, 10 minutes) once with PBS and twice more with the washing medium. Granulosa cells were then used for co-culture with the oocytes.

### IN VITRO MATURATION

Ovaries were collected at a local slaughterhouse, placed into physiological saline (0.9 percent, w/v, NaCl) with antibiotics (100 IU penicillin and 10 µg/ml streptomycin) maintained at 30-37°C, and transported to the laboratory within 2 h of slaughter. The cumulus-oocyte complexes (COC) were aspirated from small antral follicles (1-5 mm diameter) with 18-gauge 1-inch needle attached to a 5 ml disposable syringe containing modified phosphate buffered saline (PBS) supplemented with 5 percent (v/v) heat-inactivated FCS<sup>a</sup> and antibiotics (100 IU penicillin and 10 µg/ml streptomycin). Oocytes were washed three times with Hank's balanced salt solution supplemented with 5 percent (v/v) FCS (washing medium). Only oocytes with an intact-compact cumulus cells were cultured in 2 ml of maturation medium and incubated at 39°C, 5 percent CO<sub>2</sub> in air for 24 h. The basic maturation medium was M-199 with Earle's salts and 25 mM Hepes supplemented with 0.2 percent L-glutamine, 0.02 percent of an antibiotic-antimycotic solution<sup>a</sup>.

### SPERM CAPACITATION AND IN VITRO FERTILIZATION

For washing spermatozoa, 30 and 45 percent percoll solutions were prepared

by the dilution of 90 percent isotonic percoll solution<sup>b</sup>. Then, 2 ml of 30 percent percoll solution was placed on 2 ml of 45 percent percoll in a 10-ml test tube. For the preparation of capacitated spermatozoa, one 0.5 ml-straws of frozen semen was thawed in 39°C water, and 2 ml of semen were deposited on the upper layer of the percoll gradient solution. The semen was centrifuged for 15 min at 700 g. The sedimented spermatozoa displaying good motility in the bottom of tube were resuspended in 1 ml of H-TALP medium containing 0.6 percent BSA<sup>a</sup> and 100 µg/ml heparin<sup>a</sup>. Then incubated for 15 min in CO<sub>2</sub> incubator for capacitation. Then, 300 µl of the capacitated sperm suspension were placed into 1 ml of freshly prepared fertilization medium (M-199 supplemented with 10 percent FCS), containing 20-40 matured oocytes at a concentration of 1-2x10<sup>6</sup> total spermatozoa/ml and cultured for 24 h at 39°C under 5 percent CO<sub>2</sub> in air.

### EXPERIMENTAL DESIGN

#### Experiment 1

Two culture media were used for maturing immature bovine oocytes; the oocytes were matured for 24 h according to the following scheme: (1) the oocytes were cultivated in the culture medium M-199, supplemented with 20 percent FCS; (2) the oocytes were matured in the culture medium DMEM supplemented with 20 percent FCS. After culture period, all oocytes (matured and not matured) were fertilized according to the procedure described above.

#### Experiment 2

Oocytes were matured for 24 h

<sup>a</sup> Sigma Chemical Co, St Louis, Mo.

<sup>b</sup> Pharmacia LBK Biotechnology AB, Uppsala, Sweden

**Table 1.** Effect of culture medium employed during *in vitro* maturation on fertilization rate of bovine oocytes. (Efecto del medio de cultivo empleado durante la maduración *in vitro* sobre el índice de fecundación de oocitos de bovino).

Medium	Trials (number)	Oocytes (number)	Development stage (percent)			
			normal fertilized	polyspermic	degenerated	unfertilized
M-199	3	150	83 (55) <sup>a</sup>	15 (10)	7 (5)	45 (30)
DMEM	3	145	57 (39) <sup>b</sup>	17 (12)	6 (4)	65 (45)

<sup>a,b</sup>Different superscripts in the same column denote highly significant differences ( $p < 0.01$ ).

according to the following scheme: (1) the oocytes were cultured in the basic maturation medium supplemented with 20 percent FCS; (2) the oocytes were incubated in the maturation medium supplemented with 20 percent ECS; (3) the oocytes were matured in the basic maturation medium supplemented with 0.8 IU/ml PMSG plus 1.6 IU/ml hCG; (4) the oocytes were matured in the maturation medium without the addition of supplements (control group). The *in vitro* fertilization procedure was the same as in experiment 1.

#### Experiment 3

Cumulus-enclosed and cumulus-denuded oocytes were cultured for 24 h in the basic maturation medium (described above) supplemented with 20 percent FCS. Then, all oocytes were *in vitro* fertilized according to experiment 1.

#### Experiment 4

Immature bovine oocytes were co-cultured with granulosa cells ( $1 \times 10^6$  cells/ml) in the basic maturation medium supplemented with 20 percent FCS. Oocytes incubated in the basic maturation medium without co-culture with granu-

losa cells were considered as control group. After maturation period, oocytes were fertilized according to the procedure described in experiment 1.

#### OOCYTE CHROMOSOME PREPARATION

At the end of the culture period for fertilization, the oocytes were placed into 10 ml conical tubes (manufacturer) and vortex-agitated for 2 min in 5 ml of trisodium citrate (0.88 percent) and trypsin (0.02 percent) hypotonic solution. After 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 (v/v) methanol:acetic acid followed by a second solution of 3:1 (v/v) methanol:acetic acid for 24 h. Finally, the oocytes were mounted on slideglass, stained with 5 percent Giemsa and examined with the light microscope at 400 and 1500 x magnification for evaluation of maturation and fertilization.

#### CRITERIA FOR FERTILIZATION AND STATISTICAL ANALYSIS

Oocytes were morphologically

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evaluated for stage of fertilization after culture. The development stage of ova was classified as follows: (1) oocytes without both male and female pronuclei were judged as unfertilized; (2) oocytes with both male and female pronuclei and with residual sperm-tail were defined as normal fertilized; (3) oocytes with more than two pronuclei and decondensed sperm heads were considered to be polyspermic; (4) oocytes with a cytoplasm vacuolated or fragmented were considered to be degenerated. Data were statistically analysed by Chi-square test (S.A.S. Institute Inc. 1982).

### RESULTS

#### EXPERIMENT 1

The results of the fertilization rates of the oocytes matured in two different media are shown in **table I**. When oocytes were matured in M-199 medium (55 percent) the percentage of fertilized oocytes was significantly higher ( $p < 0.01$ ) than those found in the group which was matured in the DMEM (39 percent).

#### EXPERIMENT 2

The effects of the oocyte maturation in the presence of FCS, ECS and hormonal supplements on fertilizability are shown in **table II**. The oocytes matured in the M-199 with a supplement of FCS (55 percent), ECS (57 percent) or gonadotropins (60 percent) showed higher fertilization percentages ( $p < 0.001$ ) than the oocytes matured in the absence of these supplements (31 percent).

#### EXPERIMENT 3

Results of fertilization of the oocytes with or without cumulus cells are shown in **table III**. The normal fertilization rate of cumulus-enclosed oocytes (60 percent) was significantly greater ( $p < 0.001$ ) than that of denuded oocytes. The proportion of degeneration in denuded oocytes was similar to that in the cumulus-enclosed oocytes.

#### EXPERIMENT 4

The results of the fertilization rates of the oocytes matured in co-cultured with granulosa cells are showed in **table IV**. When the immature bovine oocytes were

**Table II.** Effect of different types of serum and hormone supplements employed during in vitro maturation on fertilization rate of bovine oocytes. (Efecto de diferentes tipos de suero y suplementos hormonales empleados durante la maduración *in vitro* sobre el índice de fecundación de oocitos de bovino).

Supplements	Trials (number)	Oocytes (number)	Development stage (percent)			
			normal fertilized	polyspermic	degenerated	unfertilized
FCS	3	100	55 (55) <sup>a</sup>	11 (11)	6 (6)	28 (28)
ECS	3	100	57 (57) <sup>a</sup>	10 (10)	5 (5)	28 (28)
PMSG+HCG	3	100	60 (60) <sup>a</sup>	9 (9)	4 (4)	27 (27)
CONTROL	3	105	31 (31) <sup>b</sup>	9 (8)	12 (11)	53 (50)

<sup>a,b</sup>Different superscripts in the same column denote highly significant differences ( $p < 0.001$ ).

**Table III.** Effect of the bovine oocyte type employed during *in vitro* maturation on fertilization rate of bovine oocytes. (Efecto del tipo de ovocito empleado durante la maduración *in vitro* sobre el índice de fecundación de ovocitos de bovino).

Oocyte type	Trials (number)	Oocytes (number)	Development stage (percent)			
			normal fertilized	polyspermic	degenerated	unfertilized
Cumulus-Enclosed	3	125	75 (60) <sup>a</sup>	11 (9)	5 (4)	34 (27)
Cumulus-Denuded	3	120	36 (30) <sup>b</sup>	6 (5)	7 (6)	71 (59)

<sup>a,b</sup>Different superscripts in the same column denote highly significant differences ( $p < 0.001$ ).

co-cultured with one million of granulosa cells, the percentage reaching pronuclear stage (70 percent) was significantly higher ( $p < 0.001$ ) than that found in the control group (55 percent).

## DISCUSSION

Conditions under which bovine oocytes are matured *in vitro*, such as the culture medium, the source of serum used to supplement the medium, the oocyte type and the co-culture of the oocyte with granulosa cells in the maturation medium, can affect the proportion of matured oocytes that subsequently develop to zygotes (Rose and Bavister, 1992; Harper and Brackett, 1993).

A variety of media have been used for *in vitro* maturation and culture, but TCM-199 has been used for both maturation and culture more frequently than any other medium (Berg and Brem, 1989). In this experiment, M-199 medium was clearly superior to DMEM as a maturation medium when followed by *in vitro* fertilization of matured oocytes in H-TALP. Attempting to explain the reason for M-199 being superior to

DMEM in the proportion of fertilized oocytes would be highly speculative. There are many differences in the composition of two media. The M-199 is a more complete medium than DMEM. Furthermore, M-199 contains Hepes buffer with an apparent  $P_k$  of 7.31 at 37°C, provides a maximum buffering when added to standard phosphate-bicarbonate buffered media at the optimal pH and temperature range required by most mammalian cells in culture.

The maturation medium supplemented with ECS, FCS or gonadotropin hormones supported germinal vesicle breakdown and subsequent fertilization effectively compared with M-199 alone. The results suggest that the addition of serum to the medium during oocyte maturation was responsible, at least in part, for the induction of the maturation, and subsequent fertilization of oocytes was highly dependent of some components which were in the serum. In this sense, Schroeder *et al.* (1991) found a major glycoprotein in FCS (fetuin) that inhibits zona pellucida hardening during mouse oocyte maturation and increase the rates of *in vitro* fertilization. Schellander *et al.* (1990) also reported that the beneficial

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**Table IV.** Effect of the presence of granulosa cells during *in vitro* maturation on fertilization rate of bovine oocytes. (Efecto de la presencia de células de la granulosa durante la maduración *in vitro* sobre el índice de fecundación de ovocitos de bovino).

Granulosa cells concentration	Trials (number)	Oocytes (number)	Development stage (percent)			
			normal fertilized	polyspermic	degenerated	unfertilized
1x10 <sup>6</sup> CELLS/ml	3	125	88 (70) <sup>a</sup>	6 (5)	5 (4)	26 (21)
CONTROL	3	125	69 (55) <sup>b</sup>	9 (7)	9 (7)	39 (31)

<sup>a,b</sup>Different superscripts in the same column denote highly significant differences ( $p < 0.05$ ).

effect of ECS supplement might be due to relatively high LH concentration. One mechanism by which LH may enhance *in vitro* maturation of bovine oocytes is through modifying the nutritional environment to increase the energy available for the oocyte to support its functional role in fertilization and subsequent development (Brackett and Zuelke, 1993).

Beneficial effects of cumulus cells on maturation have been reported for different domestic animal oocytes (Staigmiller and Moor, 1984; Goto *et al.*, 1988). In bovine, cumulus cells have an essential role for promoting normal cytoplasmic maturation of oocytes and for normal pronuclear formation (Mochizuki *et al.*, 1991). The results obtained in our study were similar to those reported by the authors above mentioned, confirming that cumulus cells are important for inducing the acrosome reaction of bull spermatozoa and for maintaining high fertilization rate. It is proposed that the cumulus cells surrounding the oocyte protect the zona pellucida against hardening (DeFelici and Siracusa, 1982; Katska *et al.*, 1986). We suggest that cumulus-denuded oocytes have low fertilizability due to the harde-

ning of the zona pellucida and incomplete cytoplasmic maturation due to the disconnection of junctional complexes.

Several researchers (Critser *et al.*, 1986; Fukui and Ono, 1989) 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. In our study, only one concentration (1x10<sup>6</sup> cells/ml) of granulosa cells was used, resulted in higher normal fertilization rates than in group to which no cells was added. It has been reported that although there is usually an estrogen-active follicle present every day from day 4 of the estrous cycle until ovulation (McNatty *et al.*, 1984), the ability of granulosa cells to synthesize progesterone and estradiol-17 $\beta$  differs according to follicle size, follicle health and the estrous cycle (Henderson *et al.*, 1987). Mochizuki *et al.* (1991) suggests that granulosa cells added to the culture medium must be collected from health antral follicles.

In conclusion, this study has demonstrated that the conditions of the oocyte maturation previous to the

fertilization play an important role on the fertilization rate of the matured oocytes. In this sense, M-199 medium, the addition of serum and hormonal supplements to the culture medium, the presence of

cumulus cells surrounding the oocyte, as well as the addition of granulosa cells to the maturation medium contributed to increase the fertilizability of the *in vitro* matured bovine oocytes.

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