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The Effects of Serum, Steroid and Gonadotropins on *In vitro* Maturation and Fertilization of Bovine Oocytes

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Abstract: The effect of serum, steroid and gonadotropins on maturation of bovine oocytes and subsequent embryo development were evaluated. This study was designed to investigate factors affecting IVP of bovine embryos and 16 treatment groups were formed including different combinations of FSH, LH, E2, ECS, FCS. Ovaries were obtained from cows at a slaughterhouse and transported to the laboratory in PBS at 33°C. Collected oocytes from antral follicles were cultured for 24 h in a 400 L of maturation medium (TCM-199) overlaid with mineral oil under 5% CO_2, 95% humidity in air at 38.5°C. Oocytes with expanded cumulus were transferred to the fertilization medium and coincubated with frozen-thawed and heparin treated spermatozoa for 15 h. After the incubation, oocytes were transferred to culture medium and developmental stages were recorded. Maturation rates were not significantly different among 14 treatments except control group (33%) and group 13 containing LH (66.7%). There was a low cleavage rate (35.7%) in the control group as in maturation results. Similarly, group 3 containing ECS, FSH and LH showed lowest cleavage rate (41.7%). On the contrary, highest cleavage rate (75.9%) was observed in group 14 containing LH and E2. Differences between maturation groups on cleavage rates were significant at $p<0.05$ level. Different serum and hormon supplementations to maturation media do not have any advantage against each other, but fertilization rates were clearly high in the groups supplemented with E2. Especially, supplementation of LH and E2 together in the maturation media increased fertilization rates significantly.

Key words: Bovine, oocyte, maturation, fertilization, hormone, serum

INTRODUCTION

Mammalian oocytes undergo spontaneous meiotic maturation when they are removed from antral follicles and exposed to media *in vitro* (Edwards, 1965). However, the developmental capacity of bovine oocytes matured *in vitro* was significantly lower than that of oocytes matured *in vivo*, even though the nuclear maturation rates were similar (Salustri *et al*., 1989). This is, in part, thought to be due to inappropriate cytoplasmic maturation. Therefore, maturation media have been supplemented with hormones (Moor and Trounson, 1977), growth Factors (Harper and Brackett, 1993; Lonergan *et al*., 1996), serum (Yoshida *et al*., 1992) and follicular fluid (Kim *et al*., 1996) in attempts to improve the developmental ability of oocytes.

The effect of hormonal supplementation during *In vitro* Maturation (IVM) of bovine oocytes were investigated using serum-free (Harper and Brackett, 1993; Romero-Arredondo and Seidel, 1996; Younis *et al*., 1989) and serum-containing media (Yoshida *et al*., 1992; Fukui and Ono, 1989; Fukushima and Fukui, 1985). However, conflicting data have been reported regarding the necessity and optimum combinations and concentrations of hormones and serum during *In vitro* fertilization (IVF). Improvement of developmental competence of mammalian oocytes by supplementation of IVM media with hormon and serum supplements has been the subject of many investigations. Following the early reports of Moor and Trounson (1977) and Staigmiller and Moor (1984) in which addition of granulosa cells, gonadotropins (FSH, LH) and estradiol 17β (E2) to the culture media was found to enable the sheep Cumulus Oocyte Complexes (COCs) to mature outside follicles, supplementation of the IVM media with gonadotropins and estradiol has been found to be essential for acquisition of developmental capacity of oocytes in cattle (Fukushima and Fukui, 1985; Brackett *et al*., 1989).

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Supplementation of the IVM media with a serum source like Fetal Calf Serum (FCS) (Staigmiller and Moor, 1984) or Estrus Cow Serum (ECS) (Fukushima and Fukui, 1985; Brackett *et al*., 1989) has also been found to be necessary for achieving high maturation rates for bovine oocytes.

In this study, the effects of supplementation of serum (ECS, FCS), gonadotropins (FSH, LH) and steroid (estradiol 17β) during maturation on subsequent fertilization *in vitro* were investigated.

MATERIALS AND METHODS

All chemicals, reagents, hormones, media and antibiotics were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise indicated.

Collection of ovaries: Ovaries were collected at a local slaughterhouse from cows and heifers, mostly of Holstein origin and transported to the laboratory within 2-3 h of collection in Phosphate Buffered Saline (PBS) at 30-35°C.

Collection of oocytes: Upon arrival in the laboratory, the ovaries were washed twice with fresh PBS containing 3 mg mL^{-1} BSA. All antral follicles of approximately 2-8 mm in diameter were disected with a bisturi and washed with PBS containing 3 mg mL^{-1} BSA. COCs were selected for a compact cumulus mass of at least 3 layers of cumulus cells and homogeneous cytoplasm.

***In vitro* maturation:** Selected COCs were washed 3 times in PBS containing 3mg mL^{-1} BSA and placed into 250 µL of maturation medium in 4-well plates (Nunc, Roskilde, Denmark), 25-30 oocytes per well. Oocyte maturation medium consisted of Tissue Culture Media (TCM-199) supplemented with 6 mg mL^{-1} BSA, 0.20 mM pyruvate, 50 µg mL^{-1} gentamycin and different hormon and serum combinations (Table 1). Oocytes were matured for 24 h at 38.5°C in an atmosphere of 5% CO_2 in air (Heraeus BB16, Heraeus Instruments, Hanau, Germany).

Sperm capacitation: Swim-up procedure was used for sperm preparation using Tyrode's Albumin-Lactate-Pyruvate (TALP) medium (Parrish *et al*., 1986). The composition of TALP was: 99.0 mM NaCl, 3.1 mM KCl, 25.0 mM $NaHCO_3$, 0.35 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.1 mM $MgCl_2 \bullet 6H_2O$, 21.6 mM lactic acid, 1.0 mM Na pyruvate, 6.0 mg mL^{-1} BSA-V, 10 mM Hepes and 100 mg mL^{-1} penicillin G. The pH of TALP medium was adjusted to 7.4. Frozen semen was thawed at 37°C for 30 sec and 0.2 mL aliquots were layered under 1.5 mL of TALP at 39 °C in 10 mL centrifuge tubes. The samples were incubated at 39°C and 5% CO_2 in air for 1 h. The upper 1 mL was collected, pooled into a new 10 mL centrifuge tube and centrifuged at 100×g for 10 min. The pellet was resuspended with 1 mL TALP and allowed to stand at 22°C for 5 min. An additional 3 mL TALP was added to the sperm suspension and centrifuged at 100×g for 10 min. The supernatant was pipetted off and the sperm pellet was resuspended in the remaining fluid (100 µL). This sperm suspension was diluted 1:1 with heparin solution (70 units in 2.5 mL TALP) and incubated for 15 min at 39 °C and 5% CO_2.

***In vitro* fertilization:** The matured oocytes were washed twice in TALP medium and transferred into 400 µL fertilization medium in Nunc 4-well plates (25-30 oocytes per well) under mineral oil that had been equilibrated overnight at 39°C and 5% CO_2. Fertilization medium consisted of a modified Tyrode's medium supplemented with 6 mg mL^{-1} fatty acid-free BSA and 0.25 mM pyruvate. The heparin-capacitated sperm suspension was added to the droplets to achieve a final concentration 1×10^6 mL^{-1}. The sperm and oocytes were coincubated at 39°C and 5% CO_2 for 18-20 h.

***In vitro* culture:** After fertilization, presumptive zygotes were washed twice in TCM 199, supplemented with 6 mg mL^{-1} BSA, 100 IU mL^{-1} penicillin G, 75 µg mL^{-1} streptomycin and 10 mM Hepes, transferred to pre-equilibrated 400 µL of culture medium and incubated at

Table 1: Serum, steroid and gonadotropin combinations for IVM

TCM199 with	Treatment groups															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
ECS (%10 v v^{-1})	X	X	X	X												-
FCS (%10 v v^{-1})					X	X	X	X								-
FSH (2µg mL^{-1})		X	X	X		X	X	X	X	X	X	X				-
LH (10µg mL^{-1})			X	X			X	X		X		X	X	X		-
Estradiol 17β (1 µg mL^{-1})				X				X			X	X		X	X	-

39°C and 5% CO_2 in air for 5 days without any serum supplementation or medium change. Oocytes evaluated 48 h after insemination and found to be in the two-cell stage or with pronuclei were considered to have matured and subsequently to be fertilized.

Statistical analyses: The data were analyzed by Chi-square analysis, with a probability level of $p<0.05$ being considered significant.

RESULTS AND DISCUSSION

A total of 1235 usable oocytes from 395 ovaries was collected (3.1 oocytes per ovary). The effects of serum, steroid and gonadotropins on maturation and fertilization of bovine oocytes are shown in Table 2. The maturation rates ranged between 35 and 90.7% in the treatment groups. Maturation frequency did not differ significantly among treatments except group 13 and control group ($p>0.05$, Table 2). The fertilization rates ranged between 41.7 and 75.9% in the treated groups. Oocytes incubated in maturation medium containing LH and E2 (Group 14) showed lower maturation rate (72.5%) than the others but they showed highest fertilization rate (75.9%). In contrast, oocytes incubated with ECS, FSH and LH (group 3) showed higher maturation rate (90%) than the others (except group 12), whereas fertilization was worst (41.7%) in group 3.

In the present study, we found that cumulus expansion (maturation) was not significantly affected by combinations of hormones and serum. Although the combination of LH and E2 improved cleavage rates slightly ($p<0.05$), different combinations of hormones and serum did not affect subsequent embryo development *in vitro*. An unexpected result of this study was that nearly 70% of oocytes that matured in a serum free medium containing LH and E2 were fertilized and cleaved at a rate of 90%. Subsequent treatments demonstrated that both oocyte fertilizability and embryonic viability increased in a medium containing E2. It is clear that FSH and estrogen are important components for IVM (Beker *et al*., 2002; Ali and Sirard, 2002).

Immature bovine oocytes *in vivo* remain arrested in the first meiotic prophase until maturation is induced by the interaction of steroids, gonadotropins and other follicular constituents. When oocytes are removed from antral follicles, they begin spontaneus meiotic maturation, presumably due to release from inhibitory influences of substances in follicular fluid or produced by cumulus cells through gap junctions (Karlach, 1987). When FSH was added to *in vitro* maturation medium, it appeared to stimulate cumulus cells of oocyte cumulus complexes to secrete a positive factor that could override arrest due to hypoxanthine and that could trigger meiotic resumption (Byskov *et al*., 1997). In addition, FSH stimulated an increase of cAMP concentrations and cumulus expansion Cumulus expansion occurs by synthesis of hyaluronic acid and gap junction endocytosis (Saeki *et al*., 1991). In the present study, except group 13 (LH) and control group (no supplementation), hormon and serum combinations induced sufficient cumulus expansion.

Cumulus expansion *in vivo* may facilitate sperm capacitation, normal fertilization and development of zygotes. However, several reports (Lonergan *et al*., 1996; Paula-Lopez *et al*., 1998) question the role of cumulus expansion during IVM in supporting subsequent embryo development. Further investigations are required to determine to what extent cumulus expansion is related to nuclear cytoplasmic maturation. Supplementation of gonadotropins to maturation medium has been shown to enhance the fertilizability and developmental ability of mammalian oocytes matured *in vitro*

Table 2: Effect of serum, steroid and gonadotropins during *in vitro* maturation of bovine oocytes on subsequent fertilization (Data were pooled from 3 replicates)

TCM 199 with	No. oocytes examined	No. (%) oocytes matured (cumulus expansion)	No. (%) oocytes fertilized (cleavaged)
Group 1 (ECS)	75	54/75 (72)[a]	27/54 (50)[b]
Group 2 (ECS-FSH)	77	56/77 (72.7)[a]	29/56 (51.8)[b]
Group 3 (ECS-FSH-LH)	80	72/80 (90)[a]	30/72 (41.7)[c]
Group 4 (ECS-FSH-LH-E2)	80	70/80 (87.5)[a]	40/70 (57.1)[b]
Group 5 (FCS)	75	67/75 (89.3)[a]	35/67 (52.2)[b]
Group 6 (FCS-FSH)	74	61/74 (82.4)[a]	35/61 (57.4)[b]
Group 7 (FCS-FSH-LH)	81	68/81 (84)[a]	36/68 (52.9)[b]
Group 8 (FCS-FSH-LH-E2)	78	70/78 (89.7)[a]	43/70 (61.4)[b]
Group 9 (FSH)	75	57/75 (76)[a]	28/57 (49.1)[b]
Group 10 (FSH-LH)	75	67/75 (89.3)[a]	33/67 (49.3)[b]
Group 11 (FSH-E2)	75	64/75 (85.3)[a]	32/64 (50)[b]
Group 12 (FSH-LH-E2)	75	68/75 (90.7)[a]	34/68 (50)[b]
Group 13 (LH)	75	50/75 (66.7)[b]	30/50 (60)[b]
Group 14 (LH-E2)	80	58/80 (72.5)[a]	44/58 (75.9)[a]
Group 15 (E2)	80	64/80 (80)[a]	44/64 (68.8)[b]
Group 16 (Control)	80	28/80 (35)[c]	10/28 (35.7)[d]

a,b,c: The differences among groups having different letter in same column were significant ($p<0.05$)

(Moor and Trounson, 1977; Younis *et al*., 1989; Fukushima and Fukui, 1985; Saeki *et al*., 1990). Choi *et al*. (2001) reported that gonadotropins did not improve the embryonic development. Enhanced cytoplasmic maturation of oocytes was obtained in some studies when gonadotropins were included in IVM media. In the pig, LH selectively improved cytoplasmic maturation as estimated by formation of male pronuclei (Mattioli *et al*., 1991). In cattle, FSH and LH enhanced fertilizability and developmental ability of oocytes matured *in vitro* (Romero-Arrando and Seidel, 1996). In the present study, FSH and LH did not significantly improve embryo development of bovine oocytes in IVM medium with or without serum. Keefer *et al*. (1993) reported that a very high concentrations of LH significantly decreased embryo development. In the present study, there was no effect on embryo development of LH because of low dose.

Fertilization rates (as reflected by the proportion of oocytes cleaved per oocytes inseminated) and development (as reflected by the proportions of oocytes matured and fertilized *in vitro* that reached 4-8 cell and 8-16 cell stages in culture) were enhanced by the addition of proestrous (Younis *et al*., 1989) or estrous (Schellander *et al*., 1990; Sanbuissho and Therelfall, 1989) cow sera to media employed for IVM. Sanbuissho and Therelfall (1989) reported that the addition of serum to the maturation medium was responsible for the induction of the maturation and the germinal vesicle breakdown of oocytes was highly dependent on some components which were in the serum. All the same, addition of ECS and FCS to maturation medium contributed to an inreased incidence of pronucleus formation. In our study, two types of cow serum (ECS, FCS) were added to the maturation medium with or without gonadotropins and E2 and their effect on the maturation and fertilization of oocytes was determined. It was observed beneficial effect of serum supplementation, but differences were not significantly different. Sanbuissho and Therelfall (1990) noted that the addition of ECS and FCS to the maturation medium significantly increased the maturation rates. The cumulus expansion and fertilization were not improved adding serum and no significant difference was found among groups. A significantly higher fertilization rate (75.9%) was obtained from group 14 (containg LH and E2). Schellander *et al*. (1990) noted that incidence of cleavage after IVF of oocytes matured in FCS without hormones was very low. Addition of LH and E2 increased the cleavage rate. This emphasizes the importance of hormonal supplementation of the maturation medium for aquisition of cleavage competence (Moor and Trounson, 1977). When ECS was used during maturation a high proportion of the fertilized oocytes developed to the two cell stage (Schellander *et al*., 1990). The results reported that ECS contains substances which increase the cleavage competence of *in vitro* matured and fertilized oocytes. The beneficial effect of ECS in the work might also be a result of its relatively high LH content. In contrast, we did not observe beneficial effect of ECS in the present research.

Steroids, such as estrogens, improve the completion of maturation changes in both the nucleus and cytoplasm of mammalian oocytes (Moor *et al*., 1980; Fukui *et al*., 1982). Developmental potential of bovine oocytes matured *in vitro* were increased when E2 was added to the maturation medium. It is indicated that estradiol 17β beneficial to cleavage but not maturation of oocytes. These findings are similar to the research by McGaughey (1977) who reported that E2 at 10 g mL^{-1} had an inhibitory effect on the maturation of denuded porcine oocytes. Thibault *et al*. (1975) and Moor *et al*. (1980) have also indicated that steroids play an important role in the synthesis of the presumed male pronucleus growth factor which appears in the oocyte cytoplasm during maturation *in vivo*. There is little information about possible synergetic effects of gonadotropins (LH and FSH) and steroids (estradiol, progesterone) on fertilizability of bovine oocytes. Moor and Trounson (1977) showed in sheep that the addition of estrogen to a medium containing FSH and LH resulted in the development of 26-50% to blastocysts. Fukushima and Fukui (1985) reported that the fertilization rates of bovine oocytes cultured with the addition of estradiol, FSH and LH was significantly higher than controls. These results indicate that successful maturation depends upon sequential development of follicular responsiveness initially to estrogen and FSH and then to LH. The presence of LH and E2 in the maturation medium (Group 13, 14, 15) seems important for fertilizability as was indicated by an increase in fertilization rate in the present experiment. However, addition of FSH resulted in no significant increase in fertilization ratein the present study.

CONCLUSION

IVF systems for embrio production have improved tremendously. *In vitro* fertilization rates are high and maturation conditions are close to optimal when oocytes are competent. To improve these systems, work on the quality of the immature bovine oocytes is required. The present study indicates that the addition of gonadotropins and E2 to a maturation medium would be necessary to improve the fertilizability and developmental ability of bovine oocytes *in vitro*. Ali and Sirard (2002) reported that the essential role of FSH, EGF and E2 on the

kinetics od nuclear and cytoplasmic maturation that are essential for the formation of an egg capable of fertilization and development. Although, supplementation of estradiol to a serum-free maturation medium negatively affects bovine oocyte nuclear maturation and subsequent embryo development. Although, these effects are attenuated in the presence of FSH, they strongly suggest omission of estradiol in routine maturation protocols of bovine oocytes. Differences from study to study also may be due to different IVF systems. It is also important to evaluate external factors involved in the maniplation of oocytes before fertilization and culture which can affect the expression of developmental competence. Especially, conditions of oocyte recovery and temperatures during oocyte preparation may influence the developmental competence. Oocyte quality is an important factor affecting success of IVF systems (Sirard and Blondin, 1996).

The data presented clearly demonstrate that IVM of bovine oocytes in the presence of LH and E2 not only affects nuclear maturation but also improves the developmental capacity of the oocytes as reflected by the enhanced fertilization rates.

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