EFFECTS OF OVARY TYPE, OOCYTE GRADE, HORMONE, SPERM CONCENTRATION AND FERTILIZATION MEDIUM ON *IN VITRO* MATURATION, FERTILIZATION AND DEVELOPMENT OF BOVINE FOLLICULAR OOCYTES

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Summary

In vitro embryo production (IVP) is affected by various factors during *in vitro* maturation, fertilization, and development. In this experiment, the effect of ovary type, quality of follicular oocyte, medium used for fertilization, presence of hormone in medium, sperm concentration on *in vitro* maturation and fertilization were examined for effective IVP. *In vitro* maturation was carried out using TCM-199 supplemented with 15% FCS and hormones in 5% CO_2 incubator for 24h. *In vitro* fertilization was performed with frozen-thawed sperm in modified mTALP medium containing 0.3% BSA, 10 μg/ml heparin, and 5mM/ml caffeine for 24 h. The fertilized embryos were co-cultured on monolayer of cumulus cells in TCM-199. When oocytes were collected from functionally active and inactive ovaries, maturation rate was 76.9 and 7.7%, respectively. When oocytes were classified morphologically to good and poor grades, maturation rate was 75 and 58.8%, respectively. FSH + LH + E_2 (86.4%) showed higher maturation rate than control (53.0%) and FSH (73.0%). The fertilization rate was 28.2, 100 and 91.7% in 1.6×10^5, 5.0×10^5 and 10.0×10^5 sperm concentration per ml. When oocytes were fertilized in mTALP and BO media, fertilization and cleavage rates of oocytes in mTALP were higher (84.3 and 56.9%) than those (67.4 and 23.3%) in BO medium. In this experiment, *in vitro* maturation, fertilization and development of oocytes were affected by type of ovary, grade of oocyte, hormones, sperm concentration and fertilization medium.

(**Key Words**: Follicular Oocyte, *In Vitro* Maturation, *In Vitro* Fertilization, Hormone, Sperm Concentration)

Introduction

In vitro maturation, fertilization, and culture of follicular oocytes (IVM/F/C) are effective techniques for embryo transfer, sexing, cloning, nuclear transfer and the production of transgenic animals by micromanipulation of embryos (Fayer-Hosken, 1990; Gordon and Lu, 1990). Maturation rates of 90% (Lim et al., 1992), fertilization rates of 80 to 90% (Saeki et al., 1991), and morula/blastocyst yields of 40 to 60% (Zhang et al., 1992) following *in vitro* culture of bovine follicular oocytes were recently reported.

Protocols for maturation, fertilization, and development of bovine follicular oocytes differ considerably from laboratory to laboratory. Factors affecting *in vitro* maturation include stage of cycle at which ovaries are recovered, follicular size (Tan and Lu, 1990), morphology of follicle and oocyte (Leibfried and First, 1979), presence of hormones (Sanbuissho and Threlfall, 1988; Brackett et al., 1989), and/or serum (Brackett et al., 1989) in the medium, and duration of the maturation period (Monaghan et al., 1993).

Factors influencing fertilization rate include sperm motility (Wolf et al., 1984), sperm concentration (Wolf et al., 1984; Ling and Lu, 1990; Shioya, 1992), number of oocytes per unit volume of medium (Ling and Lu, 1990), sire, ejaculation, duration for co-culture of sperm and oocytes (Rehman et al., 1993), type of medium (Younis et al., 1991), and capacitation process.

In order to establish more effective systems of bovine follicular oocyte *in vitro* culture, this study examined the influence of various factors (ovary and oocyte morphology, presence of hormones, sperm concentration and media type) on the outcome of *in vitro* maturation

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and fertilization.

Materials and Methods

Source of sperm and oocytes

The oocytes were recovered from ovaries collected from a slaughterhouse. Frozen semen was obtained from the Korean Native Cattle Improvement Center.

In vitro maturation of follicular oocytes

The ovaries were transported to the laboratory in 35-38℃ saline containing antibiotics. They were then washed and dried with tissue paper. Oocytes were aspirated from 1-7 mm follicles using an 18 gauge needle connected to a 10 ml syringe. Following sedimentation in a 15 ml tube, oocytes were located in a petri dish (Becton-Dickinson, USA).

Those oocytes with intact, unexpanded cumulus oophorous and evenly granulated cytoplasm were selected under a stereomicroscope, washed with culture medium and matured in a CO_2 incubator (5% CO_2: 95% air with high humidity at 39℃). Thirty oocytes were placed into 1 ml medium under paraffin oil in 4-well culture dishes (Multidish 4, Nunclon, Denmark). TCM-199 containing 25 mM HEPES buffer (GIBCO, USA) supplemented with heat-treated fetal calf serum (GIBCO), 0.11 mg/ml Na-pyruvate (GIBCO), 1 μg/ml FSH (Sigma), 2 IU/ml LH (Sigma), 1μg/ml E_2 (Sigma), and antibiotics was used as maturation medium.

Evaluation of nuclear status

After 24 hours culture, the oocyte-cumulus complexes were transferred to 1 ml conical tubes and vortexed for 3 minutes in Dulbecco's phosphate-buffered saline (DPBS) containing 0.2% hyaluronidase (Sigma, USA). The denuded oocytes were washed with DPBS and stained by a rapid staining method (Byun et al., 1991). The oocytes were examined under a phase-contrast microscope at 100x and 400x magnification for evaluation of nuclear status.

Sperm capacitation and *in vitro* fertilization

Following *in vitro* maturation, *in vitro* fertilization was carried out using procedures modified from those reported by Parrish et al. (1986). For capacitation, two straws (0.5 ml) of frozen semen were thawed in 35℃ water and then were pooled in a 15 ml tube to which mTALP medium (calcium ion free) of 10 ml was added. The tube was centrifuged at 833 g for 10 minutes, and the supernatant was removed. The pellet was gently resuspended with 10 ml of mTALP medium. Following incubation for 1 hour, the supernatant of 9 ml was was separated. After examining the viability of the sperm, the supernatant was centrifuged twice with added heparin at a concentration of 100 μg/ml. The supernatant of 9.5 ml was removed and the remainder was incubated for 15 minutes.

Matured oocytes were washed four times with modified mTALP fertilization medium and about 15 oocytes were placed in a 0.5 ml of fertilization medium under paraffin oil. The sperm ($5 - 10 \times 10^5$ cells/ml) were added to the medium and cocultured for 24 hours.

Modified mTALP (calcium ion free) supplemented with 0.3% bovine serum albumin (BSA) and antibiotics was used as capacitation medium. Modified mTALP containing 2 mM $CaCl_2$, 25 mM $NaHCO_3$ (Mochizuki et al., 1991), 0.3% BSA (Sigma, USA), 10 μg/ml heparin (Sigma, USA), 5 mM/ml caffeine (Sigma, USA), and antibiotics was used as fertilization medium.

Evaluation of fertilization

Using the rapid staining method described by Byun et al. (1991), oocytes with two pronuclei were classified as oocytes undergoing normal fertilization.

Results and Discussion

The effect of ovary type on *in vitro* maturation of follicular oocytes is shown in table 1.

The maturation rate (Met. Ⅱ) of the follicular oocytes collected from the active ovary was higher than that from the inactive ovary (76.9% vs 7.7%).

The effect of oocyte grade on *in vitro* maturation of follicular oocytes is shown in table 2.

TABLE 1. EFFECT OF OVARY TYPE ON *IN VITRO* MATURATION OF BOVINE FOLLICULAR OOCYTES

Type of ovary	No. of oocytes examined	Nuclear stage				
		Met Ⅰ(%)	Ana Ⅰ(%)	Tel Ⅰ(%)	Met Ⅱ(%)	Deg. (%)
Active[a]	26	4 (15.4)	2 (7.7)	0 (0)	20 (76.9)	0 (0)
Inactive[b]	13	11 (84.6)	0 (0)	0 (0)	1 (7.7)	1 (7.7)

[a] : Ovarian surface well vesiculated with many middle (3-10 mm) and large follicles (100 mm <).
[b] : Ovaries had almost no follicle or a few small follicles (2 mm >), creamy color and flat shape.

TABLE 2. EFFECT OF OOCYTE GRADE ON *IN VITRO* MATURATION OF BOVINE FOLLICULAR OOCYTES

Oocyte grade	No. of oocytes examined	Nuclear stage					
		GV (%)	Met I (%)	Ana I (%)	Tel I (%)	Met II (%)	Deg. (%)
Good[a]	24	0 (0)	3 (12.5)	3 (12.5)	0 (0)	18 (75)	0 (0)
Poor[b]	17	1 (5.9)	1 (5.9)	1 (5.9)	1 (5.9)	10 (58.8)	3 (17.6)

[a] : Oocytes with intact, unexpanded cumulus cells and evenly granulated cytoplasm
[b] : Oocytes with few cumulus cells (2 > layer) or unevenly granulated cytoplasm

The rates of Met. II in good oocytes and poor oocytes were 75.0 and 58.8%, respectively. Leibfried and First (1979), and Tan et al. (1988) reported similar results to those observed in this experiment. Leibfried and First (1979) showed that maturation rate of oocytes with more than three cumulus layers (61.8-61.9%) or less than three cumulus layers (7.7-62.5%) was higher than that with expanded cumulus layers (0-57.4%) or nude oocytes (2.6-22.5%), although there was some variation according to the appearance of the cytoplasm. Tan et al. (1988) reported that oocytes surrounded with multi-layered cumulus (77%) or with expanded cumulus cells (68%) showed higher maturation rate than oocytes with few cumulus cells (30%) or nude oocytes (36%). In this experiment, a higher maturation rate was obtained from the good oocyte complexes with evenly granulated cytoplasm and compact cumulus layers.

The effect of addition of gonadotropins and steroid hormone to TCM-199 medium on *in vitro* maturation of follicular oocytes is shown in table 3.

TABLE 3. EFFECT OF GONADOTROPINS AND STEROID HORMONE ON *IN VITRO* MATURATION OF BOVINE FOLLICULAR OOCYTES

Hormone	No. of oocytes examined	Nuclear stage					
		GV (%)	Met I (%)	Ana I (%)	Tel I (%)	Met II (%)	Deg. (%)
Control	15	1 (6.7)	4 (26.7)	2 (13.3)	0 (0)	8 (53.3)	0 (0)
FSH(10 μg/ml)	15	0 (0)	2 (13.3)	2 (13.3)	0 (0)	11 (73.3)	0 (0)
FSH(1 μg/ml) LH(2IU/ml) E2(1 μg/ml)	44	0 (0)	1 (2.3)	3 (6.8)	2 (4.5)	38 (86.4)	0 (0)

The maturation rates of follicular oocyte were 53, 73 and 86.4% in control, FSH, and FSH, LH and E_2 treatment, respectively. FSH has been shown to have a positive effect on maturation (Sanbuissho and Threlfall, 1988) and fertilization of follicular oocytes (Hensleigh and Hunter, 1985). Similarly, LH (Brackett et al., 1989; Chung et al., 1990) and E_2 (Brackett et al., 1989; Saeki et al., 1991) as well as combinations of those hormones (Sirard et al., 1988; Dominko and First, 1992) have been shown to positively effect maturation and development. In some reports, however, FSH + HCG (Sanbuissho and Threlfall, 1990) and FSH + LH + E_2 (Fukui and Ono, 1989) had no effect on *in vitro* maturation, fertilization, and development. In this experiment, a combination of FSH + LH + E_2 had the best influence on *in vitro* maturation.

The effect of sperm concentration on *in vitro* fertilization of bovine follicular oocytes is presented in table 4.

The fertilization rates of follicular oocytes were 28.2, 100 and 91.7% for sperm concentration of 1.6×10^5, 5.0×10^5 and 10.0×10^5 per ml, respectively. The sperm concentrations of 5.0×10^5 and 10.0×10^5 showed higher fertilization rates than that of 1.6×10^5. Sperm concentration has been shown to be an important factor in determining the fertilization rate of follicular oocytes (Wolf et al., 1984; Shioya, 1992).

The optimal sperm concentration varies according to the individual bull, fertilization medium, and capacitation method. Ling and Lu (1990) reported that 6.4×10^5 sperm/ml was optimal for *in vitro* fertilization. Kim et al. (1992) reported that 72.1% of fertilization rate was obtained with the considerably lower concentration of $0.5 - 1 \times 10^5$ sperm/ml.

The effect of fertilization medium on *in vitro*

fertilization of follicular oocytes is shown in table 5.

Fertilization and cleavage rates of oocytes were 67.4 and 23.3% respectively in BO medium and 84.3 and 56.9% respectively in mTALP. Many investigators have used BO or mTALP as fertilization medium and obtained comparatively good results with both. Brackett and Zuelke (1993) reported that insemination in TALP for a 24h interval led to best results. Younis et al. (1991) reported that mDM (modified defined medium) consisted of almost the same composition of BO medium resulted in better fertilization rates than mTALP in the goat. In this experiment, mTALP showed better fertilization and cleavage rates than BO. The compositions of two media are very similar, but BO has higher NaCl and glucose concentrations than those of mTALP. Sodium-lactate, hypotaurine, and epinephrine are contained in mTALP only. Hypotaurine and epinephrine have been shown to be effective to increase fertilizing capacity of hamster spermatozoa *in vitro* (Leibfried and Bavister, 1982) and increase the cleavage rate and developmental capacity of bovine oocytes following *in vitro* fertilization (Vergos et al., 1989; Miller et al., 1992). Lowitts and Biggers (1991) reported that NaCl and glucose were deleterious to the development of early stage mouse embryos.

TABLE 4. EFFECT OF SPERM CONCENTRATION ON *IN VITRO* FERTILIZATION OF BOVINE FOLLICULAR OOCYTES

sperm concentration (sperm / ml)	No. of oocytes examined	No. of oocytes		No. of oocytes fertilized (%)	No. of polyspermic oocytes (%)
		With two pronuclei	With one pronucleus		
1.6×10^5	39	11	0	11 (28.2)	1 (0)
5.0×10^5	10	9	0	10 (100)	1 (10)
10.0×10^5	12	11	0	11 (91.7)	0 (0)

TABLE 5. EFFECT OF FERTILIZATION MEDIA ON *IN VITRO* FERTILIZATION OF BOVINE FOLLICULAR OOCYTES MATURED *IN VITRO*

Fertilization medium	No. of oocytes examined	No. of polyspermic oocytes (%)	Total no. of oocytes fertilized (%)	No. of oocytes cleaved (%)
BO	43	2 (4.7)	29 (67.4)	10 (23.3)
mTALP	51	0 (0)	43 (84.3)	29 (56.9)

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