EFFECT OF GRANULOSA AND CUMULUS CELLS ON *IN VITRO* DEVELOPMENT OF BOVINE FOLLICULAR OOCYTES

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Summary

The effect of co-culture with cumulus cells and granulosa cells during maturation and development on *in vitro* developmental potency of follicular oocytes was examined. TCM-199 supplemented with 15% FCS and hormones was used as maturation medium. Sperm from frozen semen was capacitated in modified mTALP medium containing 0.3% BSA, 10 μg/ml heparin and 5 mM/ml caffeine. The fertilized embryos were co-cultured on monolayer of cumulus cells or granulosa cells in TCM-199. The embryo co-cultured with cumulus cells showed higher percentage of embryo developed to morula and blastocyst (73.3%) than the embryo co-cultured without cumulus cells (30.8%). The percentage of oocytes developed to morula and blastocyst among cleaved oocytes was significantly ($p < 0.05$) higher in the oocytes co-cultured with cumulus cells during development (62.4%) than in the oocytes co-cultured with granulosa cells during maturation and with cumulus cells during development (52.3%), and in the oocytes co-cultured with granulosa cells during development (52.8%). The results of this study indicate that co-culture of *in vitro* fertilized embryos with cumulus cells in the development medium increased the rate of embryos developed to morula and blastocyst among cleaved oocytes.

(**Key Words** : Follicular Oocyte, Co-culture, Cumulus Cells, Granulosa Cells, Blastocyst)

Introduction

Embryo produced by IVF (*in vitro* fertilization) technique from follicular oocytes has been worldwidely used for biotechnology and industry. Embryos fertilized *in vitro* can develop to blastocyst in serum and cells free chemically defined medium (Rosenkrans et al., 1993). But if *in vitro* fertilized embryos were not co-cultured with somatic cells, the rate of embryos developed to blastocyst is comparatively low (Shioya, 1992). Without co-culture with somatic cells it is difficult to overcome the 8-16 cell block (Camous et al., 1984). Bovine zygotes were co-cultured with oviductal cells (Fukui and Ono, 1989), trophoblastic vesicles (Camous et al., 1984), cumulus cells (Fukuda et al., 1990), granulosa cells, and BRL (Buffalo Rat Liver) cells (Rehman et al., 1994). Critser et al. (1986) reported that bovine follicular oocytes co-cultured with granulosa cells during maturation developed to blastocysts when they were transferred to sheep oviducts for 5 days. Fukui and Ono (1989) reported that when sera, hormones, and granulosa cells were added to culture medium, fertilization rate was affected, but maturation rate was not. The rate of blastocyst formation was highest when granulosa cells were added alone.

In this experiment, effect of co-culture with cumulus and granulosa cells during *in vitro* maturation and development on developmental potency on *in vitro* fertilized embryos was examined.

Materials and Methods

In vitro maturation

The oocytes obtained at slaughterhouse were washed

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and transported to the laboratory by submerging them in 35-38℃ saline containing antibiotics. The ovaries were washed again and wiped with tissue paper. The oocytes were aspirated from 1-7 mm follicles through 18 gauges needle connected to 10 ml syringe, settled in 15 ml tube, and relocated into a petri dish (Becton-Dickinson, USA). The oocytes with intact, unexpanded cumulus oophorus and granulated cytoplasm were selected under stereomicroscope, washed with culture medium for maturation, and cultured into CO_2 incubator (5% CO_2: 95% air with high humidity at 39℃). Groups of 30 oocytes were placed into 1 ml medium under paraffin oil in culture dish (Nunclon, Multidish 4, Denmark). TCM-199 containing 25 mM HEPES buffer (GIBCO, Cat. #380-2340AJ, Grand Island, NY) 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), 1 μg/ml estradiol (Sigma), 50 μg/ml gentamycin (Baver, gerocin, Korea), 100 IU/ml penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma) was used for maturation. After culture for 24 hours, some oocytes were stained with rapid staining method (Byun et al., 1991). Maturation was assessed with metaphase Ⅱ.

Sperm capacitation and *in vitro* fertilization

After *in vitro* maturation, *in vitro* fertilization was carried out using a modified procedure reported by Parrish et al. (1986). For the capacitation of sperm, 2 straws (0.5 ml/straw) 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 supernatant was removed. The pellet added with 10 ml of mTALP medium was incubated for 1 hour for swimming up motile sperm and then the supernatant of 9 ml was recovered. After examining viability of sperm, the supernatant was centrifuged two times. The supernatant of 9.5 ml was removed and the remainder was incubated for 15 minutes.

The 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 for fertilization.

The oocytes cultured for 24 hours were washed four times with fertilization medium and about 15 oocytes were placed in a 0.5 ml of fertilization medium under paraffin oil in a well of culture dish. The sperm ($0.5\text{-}1 \times 10^6$ sperm /ml) were added to the medium and co-cultured for 24 hours. And some oocytes were stained with rapid staining method (Byun et al., 1991) and were examined to verify fertilization (pronuclear formation).

Co-culture with granulosa and cumulus cells

Cumulus masses were collected during oocytes selection. They were washed 4 times with culture medium for development. Six to seven cumulus masses in 1 ml of development medium were incubated to form monolayer spontaneously. Thirty embryos stripped of cumulus cells and sperm by repeated pipetting were placed onto monolayer of cumulus cells in 1 ml development medium. Medium of 0.5 ml was initially exchanged at 24 hrs after incubation and thereafter at 48 hrs interval. Follicular fluid was collected from antral follicles of 10-20 mm diameter by aspiration and centrifuged at 500 g for 5 minutes. The pellet suspended with 1 ml of TCM-199 poured into the petri dish for removing oocytes and cell masses. Single-granulated cells were collected into 15 ml tube with 2 ml TCM-199 and centrifuged (500 g, 10 min). Supernatant was removed. Then granulosa cell pellets suspended with 2 ml TCM-199 and pipetted 5 times gently through 18 gauges needle with 1 ml syringe for cells separation. The cells were counted and 1×10^6 cells were incubated in 1 ml well. Medium was exchanged with the same procedure as mentioned above.

TCM-199 supplemented with 25 mM HEPES buffer, 15% heat-treated fetal calf serum, 0.11 mg/ml Na-pyruvate, 50 μg/ml gentamycin, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate was used for development. The zygotes were cultured for 8 days.

Data analysis

In table 2, the data were analyzed by Tukey's studentized range test.

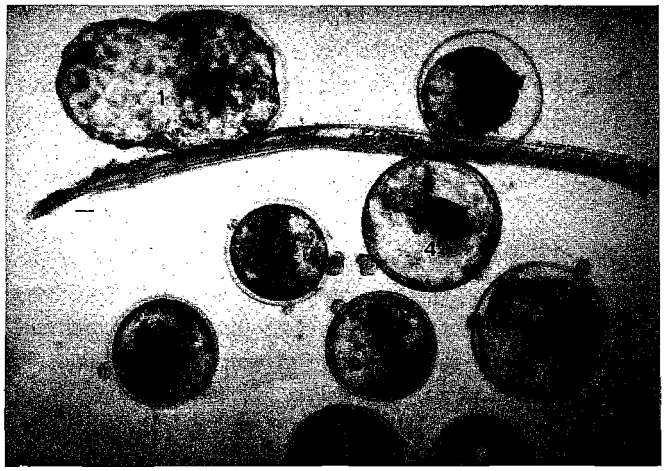
Results and Discussion

As shown in table 1, percentage of oocytes cleaved was higher in culture without cumulus cells (72.2%) than in culture with cumulus cells (41.7%). However, percentage of embryo developed from cleaved oocytes to morulae and blastocysts was lower without cumulus cells (30.8%) than in co-culture with cumulus cells (73.3%). Co-culture system was used to overcome cleavage block during *in vitro* development of embryo (Rexroad, 1989; Behboodi et al., 1991; Fukui and Ono, 1989; Mochizuki et al., 1991; Reviewed by Kane et al., 1992). Fukuda et al. (1990) indicated that a few of oocytes matured and fertilized *in vitro* developed successfully to the blastocyst stage when they were cultured in medium with or without cumulus cells. In this experiment co-culture with cumulus cells during *in vitro* development of embryo increased the percentage of embryos developed from cleaved oocytes to morulae and blastocysts.

TABLE 1. EFFECT OF CO-CULTURE WITH OR WITHOUT CUMULUS CELLS ON *IN VITRO* DEVELOPMENT OF BOVINE FOLLICULAR OOCYTES MATURED AND FERTILIZED *IN VITRO*

Cumulus cells	No. of oocytes examined	No. (%) of oocytes cleaved	No. (%) of morula and blastocysts / number of embryos cleaved
−	18	13 (72.2)	2 and 2 / 13 (30.8)
+	36	15 (41.7)	2 and 9 / 15 (73.3)

As shown in table 2, both maturation and fertilization rates of embryos showed no difference between the oocyte co-cultured with cumulus cells during development (85.9 and 81.1%) and the oocytes co-cultured with granulosa cells during maturation and with cumulus cells during development (83.8 and 80.4%). Also cleavage rate of embryo showed no difference among the three co-culture systems (42.9, 36.1 and 40.5%). However the percentage of oocytes developed to morulae and blastocysts over total and cleaved oocytes was significantly higher in the oocytes co-cultured with cumulus cells during development (27.5% and 64.2%) than in the oocytes co-cultured with granulosa cells during maturation and with cumulus cells during development (18.9 and 52.3%) and in the oocytes co-cultured with granulosa cells during development (21.6 and 52.8%). The percentage of hatched blastocyst per total blastocyst showed no significant difference among three treatments (36.4, 37.5 and 30.3%). Behboodi et al. (1991) reported that when embryos were



1 : Hatching blastocyst

3, 4, 5, 6, 7, 8, 9 : Expanded blastocysts

2, 7 : Early balstocysts

Figure 1. The blastocysts developed from *in vitro* fertilized embryos which were co-cultured on monolayer of cumulus cells during development (table 2).

TABLE 2. EFFECT OF GRANULOSA AND CUMULUS CELL CO-CULTURE DURING MATURATION AND DEVELOPMENT ON DEVELOPMENTAL POTENCY OF FOLLICULAR OOCYTES

		Maturation		Fertilization			Cleavage		Development			Hatched	
					No. of oocyte fertilized(%)					Morulae and blastocysts			
Maturation	Development	No. of oocyte examined	No. of oocyte matured (%)	No. of oocyte examined	Total	Normal	No. of oocyte cultured	No. of oocyte cleaved (%)	No. of morulae blastocysts	No. of total oocytes (%)	No. of cleaved oocytes (%)	No. of hatched blastocysts	No. of total (%) blastocysts
−	Cumulus cell	44	38 (85.9)[a]	35	30 (84.4)[a]	28 (81.1)[a]	125	48 (42.9)[a]	10/20	30/125 (27.5)[a]	30/ 48 (64.2)[a]	4/11(36.4)[a1]	
Granulosa cell	Cumulus cell	23	19 (83.8)[a]	26	23 (88.7)[a]	21 (80.4)[a]	101	36 (36.1)[a]	3/16	19/101 (18.9)[a]	19/ 36 (52.3)[b]	6/16(37.5)[a]	
−	Granulosa cell	−	−	−	−	−	264	103 (40.5)[a]	14/40	54/264 (21.6)[a]	51/103 (52.8)[b]	12/40(30.0)[a]	

Means in the same column with different superscripts differ significantly ($p<0.05$).

[1] One replication was not observed.

co-cultured with bovine oviductal epithelial cells (BOEC) and cumulus cells (CC) there was no difference between BOEC and CC in the rate of embryo developed from cleaved embryo to blastocyst (17 and 18%). The preparation of cumulus cell monolayer was simple and safe against contamination in this experiment.

It is suggested that the co-culture with cumulus cells during development is more effective than the co-culture with granulosa cells during maturation or development.

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