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소 卵胞卵의 體外成熟과 體外受精에 영향을 미치는 要因에 관한 硏究

金 相 根 · 李 晩 徽
忠南大學校 獸醫科大學

Studies on the Factors Affecting the *In-Vitro* Maturation and Fertilization of Bovine Follicular Oocytes

S. K. Kim and M. H. Lee
College of Vet. Med. , Chungnam National University

초 록

본 연구는 소의 卵胞卵의 體外成熟과 體外受精에 영향을 미치는 요인을 구명하기 위하여 미숙 난포란을 채취하여 형태적 분류를 통해 우수한 卵을 공시한 후 卵胞의 크기, 精液의 形態, 受精能獲得法, 血淸, 호르몬, 卵胞液, 卵丘細胞등을 첨가한 TCM-199 배양액에서 배양하면서 체외성숙 및 수정율을 조사하였는 바 그 결과는 다음과 같다.

1. 소 卵胞卵을 채취하여 배양을 통해 형태적 분류를 했을때 A型卵은 61.4%, B型卵은 12.1%, C型卵은 19.2%, D型卵은 4.2%였으며 發生中止 또는 退化卵은 3.0%였다. 또한 A, B, C型卵을 배양액에 배양했을때 난포란의 체외성숙율은 각각 89.1%, 78.0%, 52.6%였으며, 수정율은 각각 78.1%, 66.1%, 33.3%였다.
2. 소 卵胞의 크기를 1~2mm, 3~5mm 및 5mm이상으로 분류하여 채취한 卵胞卵의 수는 각각 67개, 98개, 63개였으며, 이를 TCM-199 배양액에서 배양했을때의 체외성숙 및 수정율은 각각 56.7% 와 44.8%, 82.5%, 71.4%와 46.0%와 28.6%였다.
3. 소 卵胞卵의 체외수정에 있어서 精巢上體 尾部精子, 稀釋精液 및 凍結精液을 이용하여 媒精하였을때 체외수정율과 분할율은 각각 63.3%, 73.3%, 70.0%와 32.7%, 37.8%, 38.3%였다.
4. 소 卵胞卵의 체외수정에 있어서 m-KRB 처리법, HIS 처리법, Ca-IA처리법, BFF처리법 및 heparin처리법으로 각각 수정능획득을 유기하였을때 체외성숙 및 분할율은 각각 53.1%, 28.1%, 33.9%와 17.7%, 50.8%와 26.2%, 48.1%와 22.8% 및 58.8%와 32.8%로서 heparin 처리법이 가장 높았다.
5. 소 卵胞卵의 체외성숙과 수정에 있어서 각 농도의 牛胎兒血淸과 FSH, HCG, β-estradiol을 첨가한 TCM-199 배양액에서 배양했을때의 체외성숙 및 수정율은 각각 76.0~82.3%와 26.2~70.0%로서 무첨가에 비해 첨가가 높았다.
6. 發情牛血淸 및 牛胎兒血淸 5~20%를 첨가한 배양액에서 배양했을때의 체외성숙율은 각각 71.7~76.9%, 74.0~80.6%였으며, 체외수정율은 51.9~58.0%와 26.2~30.0%로서, 체외수정율의 경우 發情牛血淸의 첨가가 牛胎兒血淸의 첨가에 비해 높았다.
7. 卵胞液 20~30%를 첨가한 배양액에서 배양했을때의 卵胞卵의 체외성숙율은 각각 68.0%와 64.6%, 수정율은 각각 59.6%와 60.4%로서 卵胞液 10%, 50%를 첨가한 배양액에서 배양시의

체외성숙율과 수정율에 비해 높았다.

8. 1×10^6/ml의 卵丘細胞를 첨가한 배양액에서 배양했을때의 卵胞卵의 체외성숙율과 수정율은 각각 76.5%와 61.7%로서 FCS 10%와 $1\times10^4\sim10^5$/ml와 1×10^8/ml의 卵丘細胞를 첨가한 배양액에서 배양시의 체외성숙율과 수정율에 비해 높았다.

INTRODUCTION

In recent years, the success of *in-vitro* maturation and fertilization of oocytes in animals has been greatly improved, pregnancies or offspring being obtained after culture of oocytes *in-vivo* or *in-vitro* and transfer of embryos to recipient animals. However, the percentage of oocytes reaching the blastocyst stage in a completely *in-vitro* system is still low, at 6~20%.

Some improvements of the maturation and fertilization system in oocytes have been made, but the development of reliable system to support embryonic development beyond the morula stage after *in-vitro* maturation and fertilization of oocytes is still required. They include coculturing embryos with somatic cells, oviduct epithelial cells, cumulus cells, addition of media with hormone and serum.

Recently as the industrial use of embryo transfer is possible, the researches for *in-vitro* fertilization are also advanced. So it was reported that calf was produced by embryo transfer which was fertilized *in-vitro*. However, to succeed in doing *in-vitro* fertilization requires the maturation of oocytes, capacitation of sperms, acrosome reaction, and the same conditions as environment of oviduct, but at present our true circumstances are that perfect technique in *in-vitro* fertilization is not established.

If a great deal of fertilized egg is produced by *in-vitro* fertilization after maturing follicular oocytes selected from cow in good inherited characteristics through *in-vitro* culture, it is expected that this greatly contributes technical development and study of the production of twin, clone animals and chimera animals, production of new species by gene transfer as well as animal improvement by embryo transfer, production of specfic species and use of pedigree conservation.

Since Edward(1965) reported *in-vitro* fertilization of bovine follicular oocytes in 1965, many researches about this field have been carried out. These researches are divided into several parts ; *in-vitro* fertilization with spermatozoa capacitated at *in-vitro*, with spermatozoa capacitated at *in-vivo*, and with transplantation of embryos *in-vitro* fertilization with oocytes matured *in-vitro*.

But, the research on immatured bovine follicular oocytes made poor progress because of difficulty of security of good follicular oocytes, and in adquate capacitation method of spermatozoa. Also the condition of *in-vitro* incubation was not established so that abnormal development and *in-vitro* cell block etc took place frequently.

When the results of the study so far were examined, it was known that there were many inhibiting factors affecting the *in-vitro* maturation and fertilization rate of bovine follicular oocytes. The *in-vitro* maturation and fertilization rate of bovine follicular oocytes differ with the morphology of oocytes, incubation method, and incubation condition. It was reported that the *in-vitro* maturation and fertilization rate of bov-

ine follicular oocytes varied with the size and morphosis of collected follicular oocytes, but that there was no difference in the *in-vitro* fertilization rate according to the size of follicular oocytes.

In order to solve the problems, a variety of foundmental researches were carried out. According to the recent study, a change of the composition of culture media and incubation condition, which was undergone by adding the various kinds of the hormones and serums to culture media for *in-vitro* maturation, caused the increase of the *in-vitro* maturation and fertilization rate. Also, there is research to improve the capacitation method and the type of sperms in the *in-vitro* fertilization, and to increase the *in-vitro* maturation and fertilization rate of bovine follicular oocytes by adding a variety of materials such as inactivated follicular fluids to culture media.

There is not only a wide difference between author's views, but also fragmentary research commands absolute majority. Thus, this experiment was carried out in order to investigate the rates of the maturation and fertilization of oocytes *in vitro* according to the size and morphology of follicular oocytes, semen types, capacitation method of sperm, serum, the addition of follicular fluid and cumulus cells, which are affecting the *in vitro* maturation and fertilization of bove follicular oocytes.

MATERIALS AND METHODS

1. Materials

1) Recovery of follicular oocytes

Overies were taken from Korean native cow (body weight 270~500kg) at a local abattoir, then were transported to the laboratory within 2 hrs in saline at 38℃ containing 100 IU/ml of penicillin G and 100μg/ml of streptomycin sulfate. Follicular fluids aspirated from follicles of 2~5mm in diameter through an 18 guage needle, and oocytes were collected from the fluid under a streomicroscope then washed 3 times with the culture media.

2) Isolation of estrous cow serum(ECS)

The blood was obtained from the jugular vein of estrous cow and was centrifuged at 2,000 rpm for 10 min twice. After the supernatant was filtered with 0.2μm millipore filter, and it was inactivated for 30 min at 56℃.

3) Preparation of bovine follicular fluid(BFF)

BFF collected from follicles lager than 10 mm in diameter and was centrifuged at 2,000 rpm for 10 min. The supernatant was filtered, heat-inactivated then stored in −20℃.

4) Preparation of cumulus cell

Transparent follicle of 5~10 mm in diameter were taken and the surrounded interstitial tissues were removed using a micropipette under the streomicroscope. Cumulus cell was collected in tube, centrifuged at 500 rpm for 5 min, and the supernatant was discarded. Then the cell were washed with TCM-199 containing 10% FCS twice, counted and then transferred into the culture media.

5) Culture media

The basal culture media for maturation and fertilization of oocytes *in vitro* are TCM-199 (Whittaker, M. A., Bioproducts Co. USA) which contained 10%(v/v) of FCS, 1μg/ml of FSH(Sigma, USA), 2 IU/ml of HCG, 1μg/ml of β-estradiol(Sigma, USA), 100 IU/ml of penicillin G and 100μg/ml of streptomycin sulfate. They were filtered and pasteurized with 0.2μm millipore filter before use.

In order to investigate the effect of addition of serum, BFF, and cumulus cells to *in vitro* maturation and fertilization rate, the foundmental culture media were treated as follows;

FCS and ECS were added in 5%, 10%, 15%, and 20%(v/v), BFF, 10%, 20%, 30%, and 50% (v/v), and cumulus cells, 1×10^4/ml, 1×10^5/ml, 1×10^6/ml, and 1×10^8/ml.

2. Methods

1) *In-vitro* maturation of follicular oocytes

A 50 μl drop of the culture media was covered with mineral oil(Squibb Co., USA), equilibrated in a CO_2 incubator(5% CO_2, 95% air, 38.5℃) for 5~6 hours before 2~3 hrs in incubation. The follicular oocytes were immersed 5 per drop and incubated for 24hrs.

2) Capacitation of spermatozoa

(1) Heparin treatment

Frozen semen was thawed at 38℃ for 1 min, pooled and prepared for sperm capacitation. A 2ml aliquot of the semen, which thawed was diluted 1 ml modified Tyrode's Ca-free capacitation medium in a tube, swimmed up for 1 hr at 38℃ in 5% CO_2 in air. After incubation, the top 0.8 ml of medium from each tube was pooled and the spermatozoa were washed twice (500g, 10 min) with the capacitation medium. The final pellet of semen was resuspended to 50×10^6 spermatozoa/ml containing 10 ug heparin/ml and incubated in CO_2 incubator for 15 min.

(2) BFF(bovine follicular fluids) treatment

Bovine follucular fluids was collected from follicles larger than 10~20 mm in diameter and was centrifuged at 600 rpm for 10 min. The supernatant was filtered and inactivated for in 30 min at 56℃. A 0.2 ml aliquot of the semen was diluted with 3 ml of BFF and preincubated for 4 hrs.

(3) HIS(high ionic strength) treatment

A 0.1 ml aliquot of the semen was diluted with 2ml of HIS(Brackett, 1982), incubated for 5 min 38℃, and centrifuged at 330 rpm for 5 min. The semen sediment was diluted with 1ml of BO(Brackett and Oliphant, 1975), and preincubated for 4 hrs.

3) *In-vitro* fertilization

The follicular oocytes were washed with the fertilization medium 3 times after incubation, and the adjacient cumulus cell was partially eliminated by pipetting. The oocytes were placed 5 each in a drop(45 ul) of fertilization medium which covered with mineral oil, and the capacitated sperm(1.5×10^6/ml) was then added to the oocytes. Oocytes and spermatozoa were incubated together for 20~24 hrs at 38℃ in a CO_2 incubator.

4) Judgement of maturation and fertilization

Follicular oocytes were recovered, incubated for 24 hours, and treated with 0.1% hyaluronidase(Sigma, USA) to remove cumulus cells. The follicular oocytes were fixed on a slide glass with 25% acetic acid solution for 24~48 hrs, and stained with 1% acetic-orcein solution. The rate of maturation and fertilization was determined according to those descrived by Shea *et al.* (1976) and Ball *et al.* (1984).

RESULTS AND DISCUSSION

1. Morphology of the follicular oocytes

The follicular oocytes were classified according to the method of Hanada(1985) as shown in Table 1. And their rates of *in-vitro* maturation and fertilization were listed in Table 2.

Hanada(1985) classified the follicular oocytes morphologically by the presence and growth of cumulus cell layer attached to zona pellucida. Among total 1,256 oocytes tested A type was 61.5%, B type 12.1%, C type 19.2%, and D type 4 2%.

This result is more or less similar to that reported by Hanada(1985)'s who reported that the ratio of A, B, C, and D type was 57.2%, 9.

Table 1. Morphological classification of bovine follicular oocytes recovered from an ovarian follicle

No. of oocytes examined	Oocytes morphological classification (%)			
	A	B	C	D
1256	61.4	12.1	19.2	4.2

A : Oocytes with compact, dense cumulus cells

B : Partially naked oocytes with thin cumulus layer

C : Oocytes with foggy cumulus cell and incompletely attached zona pellucida

D : Naked oocytes

Table 2. *In-vitro* maturation and fertilization rate of bovine oocytes classified by cumulus cells

Grade of oocytes	No. of oocytes examined	No. of oocytes matured(%)*	No. of oocytes fertilized(%)**
A	64	89.1	78.1
B	59	78.0	66.1
C	57	52.6	33.3

* : The number of oocytes matured to the second metaphase

** : The number of oocytes fertilized

6%, 25.7%, and 7.5%, respectively. The rates of *in-vitro* maturation which obtained from A, B, and C type oocytes by morphological classification were 89.1%, 78.0%, and 52.6%. And those of *in-vitro* fertilization rate were 78.1%, 66.1%, and 33.3%, respectively.

These results indicate that getting the best *in-vitro* maturation and *in-vitro* fertilization rate of immatured follicular oocytes requires incubation after selection by morphological classification.

2. Follicle size

When the follicular oocytes were classified into 1~2 mm, 3~5 mm, and over 5 mm in diameter and cultured with TCM-199 medium, the rates of *in-vitro* maturation and fertilization obtained were as shown in Table 3.

Number of the selected oocytes which sized 1~2 mm in diameter was 67, 3~5 mm 98, and over 5 mm 63 from total 228. The rates of *in-vitro* maturation and fertilization were 56.7%, 82.5%, and 46.0%, and 44.8%, 71.4%, and 28.6%, respectively. The value was higher for the size of 3~5 mm. Leibfried and First(1980), Fukui *et al.* (1982) and Leibfried-Rutledge *et al.* (1985) reported that the rates of the maturation and fertilization *in vitro* were high for the follicle

Table 3. Effects of follicles size on *In-vitro* maturation and fertilization rate of bovine oocytes

Size of follicles	No. of oocytes cultured	No. of oocytes matured(%)*	No. of oocytes fertilized(%)**
1~2 mm	67	38(56.7)	30(44.8)
3~5 mm	98	81(82.5)	71(71.4)
< 5 mm	63	29(46.0)	18(28.6)

* : The number of oocytes matured to the second metaphase

** : The number of oocytes fertilized

size of 3~6 mm, although significance was not recognized.

3. Semen types

The rates of fertilization and cleavage *in vitro* according to the types of semen were of bovine oocytes matured in *in-vitro* were shown as in Table 4.

The rates of fertilization and cleavage *in vitro* according to the types of semen were 63.3% and 32.7% for that epididymal cauda, 73.3% and 37.8% for the diluted semen, and 70.0% and 38.3% for the frozen semen, respectively.

The fertilization rate for the sperms of epididymal cauda was slightly lower than the result of 71.0% by Ball(1983) who employed different capacitation method. However, the value for the frozen semen was markedly higher than the result 44.0% reported by Bondioli and Wright(1983).

4. Capacitation method of sperms

The fertilization and cleavage rates of oocytes *in vitro* according to the capacitation methods of sperms were as shown in Table 5.

The rates of fertilization and cleavage *in vitro* according to the capacitation methods of sperms were 53.1% and 28.1% for m-KRB method, 9% and 17.7% for HIS method, 50.8% and 26.2% for Ca-IA method, 48.1% and 22.8% for BFF method, and 58.8% and 32.8% for heparin method, respectively. The fertilization rates above 50% were obtained from m-KRB, Ca-IA and heparin method, among which the heparin method represented the highest value.

These values were lower than 79.0% by heparin method reported by Parrish *et al.* (1984), 21~67% by m-KRB method(Iritani and Niwa. 1977; Iritani *et al.*, 1984), and 25.6~55.0% of average cleavage rate by Ca-IA method(Hanada, 1985). But these were relatively high in comparison with 40.0% by HIS method reported by Brackett *et al.* (1982), and 46.2% by BFF

Table 4. Effects of semen types on *In-vitro* fertilization of bovine oocytes matured *In-vitro*

Types of semen	No. of oocytes cultured	No. of oocytes fertilized(%)*	No. of oocytes cleaved(%)**
SEC	49	31(63.3)	16(32.7)
Neat	45	33(73.3)	17(37.8)
Frozen	60	42(70.0)	23(38.3)

SEC : sperms of epididymal cauda

* : No. of oocytes fertilized / No. of oocytes cultured

** : No. of oocytes cleaved / No. of oocytes fertilized

Table 5. Effects of capacitation methods of sperms on *In-vitro* fertilization rate of bovine oocytes

Capacitation method of sperms	No. of oocytes cultured	No. of oocytes fertilized(%)	No. of oocytes cleaved(%)
m-KRB method	64	34(53.1)	18(28.1)
HIS method	62	21(33.9)	11(17.7)
Ca-IA method	65	33(50.8)	17(26.2)
BFF method	79	38(48.1)	18(22.8)
Heparin method	67	40(58.8)	22(32.8)

* : No. of oocytes fertilized / No. of oocytes cultured

** : No. of oocytes cleaved / No. of oocytes fertilized

method(Fukui, *et al.*,1983). Especially heparin method has the best fertilization rate, which indicates, I think, that heparin at *in-vivo* follicular fluids and uterine fluids, and glycosaminoglycans(GAG) containing hyaluroic acid promote acrosome reaction and have a potent capacitation(Ball, *et al.*,1981; Bondioli and Wright,1983; Parrish *et al.*,1986).

5. Addition of hormones

The rates of maturation and fertilization *in vitro*, when the oocytes were cultured in TCM-199 medium which contained a various concentration of FCS and hormones(1μg/ml of FSH, 2 IU/ml of HCG, 1 μg/ml μ-estradiol), were as shown in Table 6.

There were no distinctive differences in the maturation rates with and without a various concentration of FCS and hormones, but the fertilization rates were higher in case of addition(26.2~70.0%) than no addition(26.2~30.0%). And the results indicate that the addition of 15% and 20% FCS and hormones was favorable for the fertilization.

These results were in accord with Shalgi (1979), Ball *et al.*(1983), and Hensleigh and Hunter *et al.*(1985), reported that the fertilization rate increased by the addition of FCS, FSH, and HCG. Fukui *et al.* (1983) suggested that FSH and HCG were not indispensible for *in vitro* maturation.

6. The concentration of FCS and ECS

The rates of the maturation and fertilization *in vitro*, when the oocytes were cultured in TCM-199 medium which contained a various concentration of ECS and FCS, were as shown in Table 7.

The maturation rate of oocytes *in vitro* cultured in TCM-199 medium with 10% ECS group(76.9%) was superior to 5% ECS group (74.5%), 15% ECS group(76.0%) and 20% ECS group(71.7%), and the fertilization rate were 15% ECS group(58.0%) was superior to 5% ECS group(55.3%), 10% ECS group(51.9%) 20% ECS group(57.8%).

The maturation rate of oocytes *in vitro* cultured in TCM-199 medium with 15% FCS group(80.6%) was superior to 5% FCS group (77.8%), 10% FCS group(78.6%) and 20% FCS group(74.0%), and the fertilization rate were 20% FCS group(30.0%) was superior to 5% FCS

Table 6. Effects of hormones added to culture media on *In-vitro* maturation and fertilization rate of bovine follicular oocytes

Concentration of serum	Hormones*	No. of oocytes examined	No. of oocytes matured(%)**	No. of oocytes fertilized(%)***
5% FCS	−	45	35(77.8)	13(28.9)
	+	54	41(76.0)	11(26.2)
10% FCS	−	42	33(78.6)	11(26.2)
	+	60	49(81.6)	42(70.0)
15% FCS	−	47	38(80.6)	14(29.8)
	+	45	37(82.2)	30(66.7)
20% FCS	−	50	37(74.0)	15(30.0)
	+	51	42(82.3)	35(68.6)

* : FSH : 1 μg/ml, HCG : 2IU/ml, β-estradiol : 1 μg/ml

** : The number of oocytes matured to the second metaphase

*** : The number of oocytes fertilized

Table 7. Effects of a various concentration of ECS and FCS added to culture media on *In-vitro* maturation and fertilization rate of bovine follicular oocytes

Concentration of serum	No. of oocytes examined	No. of oocytes matured(%)*	No. of oocytes fertilized(%)**
5% ECS	47	35(74.5)	26(55.3)
10% ECS	52	40(76.9)	27(51.9)
15% ECS	50	38(76.0)	29(58.0)
20% ECS	45	32(71.7)	26(57.8)
5% FCS	45	35(77.8)	13(28.9)
10% FCS	42	33(78.6)	11(26.2)
15% FCS	47	38(80.6)	14(29.8)
20% FCS	50	37(74.0)	15(30.0)

* : The number of oocytes matured to the second metaphase.

** : The number of oocytes fertilized

group(28.9%), 10% FCS group(26.2%) 15% FCS group(29.8%).

The results were similar to those of Sanbuissho and Threlfall(1985), Xu *et al.* (1987) and Lu *et al.* (1987), who indicated that the addition of ECS improved the rates of the maturation and fertilization. On the other hand, Fukui and Ono(1989) reported that FCS resulted in relatively higher rates of maturation and fertilization than ECS. result that FCS had higher maturation and fertilization rate than ECS.

7. Addition of BFF

The rates of the maturation and fertilization *in vitro*, when the oocytes were cultured in TCM-199 medium containing 10% FCS and matured cumulus cells were as shown in Table 8.

When the oocytes were cultured in TCM-199 medium containing BFF, the rates of the maturation and fertilization were 67.9% and 54.7% for 10% addition of BFF, 68.0% and 59.6% for 20% addition of BFF, 64.6% and 60.4% for 30% addition of BFF, and 58.8% and 54.9% in 50% addition of BFF.

This study reflected that the maturation rate of oocytes in TCM-199 medium was increased in TCM-199 addition of BFF than non-addition of BFF. Stone *et al.* (1978) reported that the

Table 8. Effects of a various concentration of BFF added to culture media on *In-vitro* maturation and fertilization rate of bovine follicular oocytes

Concentration of BFF	No. of oocytes examined	No. of oocytes matured(%)*	No.of oocytes fertilizes(%)**
10% FCS 10%	53	36(67.9)	29(54.7)
" 20%	47	32(68.0)	28(59.6)
" 30%	48	31(64.6)	29(60.4)
" 50%	51	30(58.8)	28(54.9)

* : The number of oocytes matured to the second metaphase

** : The number of oocytes fertilized

inhibiting effect of BFF could be reduced as ovarian follicles developed, and Tsafriri *et al.* (1982) reported that the composition of BFF changed according to the size of ovarian follicles and the maturation of cumulus cells.

8. Addition of cumulus cells

The rates of the maturation and fertilization *in vitro*, when the oocytes were cultured in TCM-199 medium containing 10% FCS and matured cumulus cells were as shown in Table 9.

When the oocytes were cultured in TCM-199 containing 10% FCS and matured cumulus cells, the *in-vitro* maturation and fertilization rate were 75.0% and 47.9% for 1×10^4/ml cumulus cells, 71.4% and 55.1% for 1×10^5/ml, 76.5% and 61.7% for 1×10^6/ml, and 73.0% and 61.5% for 1×10^8ml.

These results agreed to those of Ball *et al.* (1983) and Crister *et al.*(1986), who reported that the addition of cumulus cell to culture media improved the rates of *in-vitro* maturation and fetilization. On the other hand, Tsafriri *et al.* (1976) and Hillensjo *et al.*(1976, 1981) suggested that the certain inhibiting substances in BFF could repress the maturation of follicular oocytes. Nekola and Smith(1974), and Jagiello *et al.*(1977) reported that the maturation was not repressed in case of coculture with cumulus cells.

When the oocytes were cultured in TCM-199 containing 10% FCS and matured cumulus cells, the *in-vitro* maturation and fertilization rate were 75. 0% and 47.9% for 1×10^4/ml cumulus cells, 71.4% and 55.1% for 1×10^5/ml, 76.5% and 61.7% for 1×10^6/ml, and 73.0% and 61.5% for 1×10^8/ml.

These results agreed to those of Ball *et al.* (1983) and Crister *et al.* (1986), who reported that the addition of cumulus cell to culture media improved the rates of *in-vitro* maturation and fertilization. On the other hand, Tsafriri *et al.* (1976) and Hillensjo *et al.* (1976, 1981) suggested that the certain inhibiting substances in BFF could repress the maturation of follicular oocytes. Nekola and Smith(1974), and Jagiello *et al.*(1977) reported that the maturation was not repressed in case of coculture with cumulus cells.

SUMMARY

This study was carried out in order to elucidate the inhibiting factors affecting the *in-vitro* maturation and fetilization rate. Through the morphological classification the excellent oocytes were selected, and the *in-vitro* maturation and fertilization rate were investigated according to the size of ovarian follicle, semen types, capacitation method of sperm and the composition of culture media which added

Table 9. Effects of a various concentration of cumulus cell added to culture media on *In-vitro* maturation and fertilization rate of bovine follicular oocytes

No. of cumulus cell		No. of oocytes examined	No. of oocytes matured(%)*	No. of oocytes fertilized(%)**
10% FCS	1×10^4/ml	48	36(75.0)	23(47.9)
"	1×10^5/ml	49	35(71.4)	27(55.1)
"	1×10^6/ml	47	36(76.5)	29(61.7)
"	1×10^8/ml	52	38(73.0)	32(61.5)

* : The number of oocytes matured to the second metaphase

** : The number of oocytes fertilized

serum, hormones, BFF, and cumulus cell.

The results obtained are as follows;

1. When bovine follicular oocytes were selected, incubated, and classified morphologically, the distribution of them was of 61.4%, 12.1%, 19.2%, and 4.2% for A type, B type, C type, and D type, and degenerated oocytes was of 3.0%. When A, B, and C type were incubated in the culture media, the *in-vitro* maturation rate of bovine follicular oocytes was 89.1%, 78.0%, and 52.6% respcetively, and *in-vitro* fertilization rate was 78.1%, 66.1%, and 33.3%, respectively.
2. The number of bovine follicular oocytes, which were divided into 1~2mm, 3~5mm, and over 5mm, was 67, 98, and 63, respectively. When the classified bovine follicular oocytes were incubated in TCM-199, the *in-vitro* maturation rate was 56.7%, 82.5%, and 46.0%, and the *in-vitro* fertilization rate was 44.8%, 71.4%, and 28.6%, respectively.
3. When the sperms of epididymal cauda, the diluted semen, and frozen semen were used to inseminate, the *in-vitro* fertilization rate was 63.3%, 73.3%, and 70.0%, and the cleavage rate was 32.7%, 37.8%, and 38.3%, respectively.
4. When the heparin method, BFF method, and HIS method were used for the capacitation, the *in-vitro* fertilization rate was 70.0%, 53.8%, and 34.2%, and the cleavage rate was 38.3%, 23.1%, and 17%. There was nothing higher than heparin method.
5. When oocytes were incubated in the TCM-199 containing a various concentration of FCS, FSH, HCG, and β-estradiol, the *in-vitro* maturation and *in-vitro* fertilization rate were 76.0%~82.3% and 26.2%~70.0%, and so these rates were higher in comparison with no addition.
6. When bovine follicular oocytes were incubated in TCM-199 culture media that were added 5~20% of ECS, the *in-vitro* maturation and fertilization rate were 71.7% and 76.9%, respectively. When 5~20% of FCS was added TCM-199, the *in-vitro* maturation and *in-vitro* fertilization rate were 74.0~80.6% and 26.2~30.0%, respectively. Thus the *in-vitro* fertilization rate was higher in the addition of ECS than in the addition of FCS.
7. When the bovine follicular oocytes were incubated in the culture media added 20~30% of BFF, the *in-vitro* maturation rate was 68.0% and 64.6%, and fertilization rate 59.6% and 60.4%, respectively. These values for *in-vitro* maturation and *in vitro* fertilization rate indicated that 20~30% of addition was higher in comparison with 10% and 50% of addition of BFF.
8. When the follicular oocytes were incubated in culture media added 1×10^6/ml of matured cumulus cell, the *in-vitro* maturation and *in-vitro* fertilization rate were 76.5% and 61.7%, which were higher than culture media which contained 10% of FCS, and 1×10^4~10^5/ml and 1×10^8/ml of cumulus cell.

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