韓國受精卵移植學會誌(1994) 第9卷 第1號
Korean J. Emb. Trans. (1994) Vol.9, No.1 pp.103~110

# Studies on the Improvement of Performance and Reproductive Efficiency in Farm Animals V. Studies in *In Vitro* Fertilization of Follicular Oocytes in Cattle

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# 家畜의 改良 및 繁殖效率 增進에 關한 硏究 V. 소에 있어서 卵胞卵의 體外受精에 關한 硏究

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## 적 요

소 卵胞卵의 體外成熟率과 體外受精후 卵割率을 향상시키기 위하여 성숙배양액에 FCS 添加效果를 조사하였으며 FCS의 첨가수준은 5~20%였고 사용된 基本培養液은 DM(−BSA), Ham's F10 및 TCM199이었다. 卵胞直徑 2~6mm 난포로부터 채란된 卵胞卵을 39℃ 배양기에서 28시간 성숙시킨후 동결융해정자 또는 비동결정자로 體外受精시켰다. FCS 無添加區보다는 添加區에서 成熟率이 향상되었으나 FCS 첨가의 DM(−BSA)와 Ham's F10간에, 그리고 FCS 添加水準間에는 成熟率의 차이가 없었다. FCS의 添加로 성숙율은 향상되었으나 卵割率에는 영향이 없었다. 체외수정 卵胞卵의 卵割率의 卵割率이 HIS 處理後 6시간 前培養한 동결정자와의 受精에서는 현저히 낮았으나 2시간 전배양과 정자에 의해서는 향상되었고 비동결정자이용시 더욱 向上되었다. FCS 添加된 Ham's F10과 TCM199에서 成熟시킨 卵子의 卵割率이 DM(−BSA)+FCS 보다 높았다. 本 硏究 結果에서 卵胞卵의 體外成熟이 FCS첨가로 개선되었으나 卵割率에는 영향이 없었으며 卵割率이 精子處理方法에 따라 차이가 많았고 Ham's F10과 TCM199에 FCS의 添加가 보다 효과적이었다.

## INTRODUCTION

Embryo transfer(ET) in cattle has been commerically developed in recent years. ET techniquies are greatly responsible for the rapid genetic progress of productive ability and have allowed progress to be made toward increasing the number of offspring produced from genetically superior females. However, new approaches are required to enhance the economical efficiency of ET industry and the new altemative applications of ET techniques. Also, the production of embryos in large quantities for transfer remains a major problem. Recently, the *in vitro* fertilization(IVF) of follicular oocytes matured *in vitro* has been doconsiderably interested in the new approaches and developments of ani-

These studies were conducted under a grant ('87 project) from the Ministry of Education, the Republic of Korea.

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mal biotechnology and in the another techniques that may prove useful for obtaining large quantities of embryo for transfer(Iritani, 1987). These studies were conducted under a grant(87 project) from the Ministry of Education of the Republic of Korea. The bovine oocytes matured *in vitro* have been successfully fertilized under *in vitro* conditions(Iritani and Niwa, 1977;Bondioli and Wright, 1983;Fukui *et al.*,1984) and the optimum culture conditions of these oocytes have been developed(Parrish *et al.*,1986). However, the results of IVF have shown the variable success and early embryonic developmental potential to blastocyst after IVF was still greatly low in many reports(review by Wright and Bondioli, 1981). Bovine oocytes have been matured in a variety of media ranging from simple balanced salt solution (Leibfried-Rutledge, *et al.*, 1987) to complex media(Leibfried and First, 1979). Recent studies have shown that serum provided a superior environment for the bovine oocytes maturation(Leibfried-Rutledge *et al.*,1986;Sanbuissho and Threfall, 1989) and appeared to improve the fertilizability of oocytes (Schroeder and Epping,1984;Leibfried-Rutledge *et al.*,1986; Choi *et al.*,1987; Vanderhyden and Armstrong, 1989) when compared with those matured with BSA.

The present study was designed to investigate the effect of fetal calf serum(FCS) addition to different media on the *in vitro* maturation rate of bovine follicular oocytes and the cleavage of oocytes after IVF with bull spermatozoa capacitated *in vitro*.

## MATERIALS AND METHODS

### 1. Collection and *in vitro* maturation of oocytes

Ovaries of Korean cattle were obtained from a slaughterhouse and transferred at about 35℃ in saline to laboratory. The follicles ranging from 2 to 6mm in diameter were aspirated using an 18-gauge needle within 2h to 3h after salughter. Oocytes from each ovary were pooled and washed three times in fresh maturation medium. Only oocytes with an intact, compacted cumulus were then divided randomly into several groups and the 5 to 10 oocytes per petri dish were transferred to 0.4ml maturation medium perincubated under mineral oil. The oocytes were cultured for 28h at 39℃ under atmosphere of 5% $CO_2$, and 95% air with high humidity. The maturation of oocytes was determined on the basis of nuclear maturation(metaphase Ⅱ). The maturation medium for the control groups was the defined medium(DM) described by Brackett and and Oliphant(1975) and Ham's F10 prepared from powder by adding sterile distilled water, respectively and a modified DM(−BSA) and Ham's F10 for the treated-groups was supplemented with 5 to 20%(v/v) fetal calf serum (FCS) instead of BSA. All media were prepared for each experiement and were sterilized by passing through a 0.25$\mu$m membrane and gassed with a mixture of 5% $CO_2$ in air before use.

### 2. Preparation of spermatozoa

For each replicate two frozen 0.5ml straws from different bulls were thawed in a 35℃ water bath for 1 min and were treated according to the procedure of Brackett *et al.*(1982). the thawed semen were diluted with 3 ml of DM and washed by centrifugation for 5 min at 250×g. Following supermatant removal, the sperm pellets were resuspended in 3 ml of DM or high ionic strength(HS)medium and were incubated for 10 min. After this incubation, the sperm suspension was recentrifuged, resuspended with 0.8 ml of DM, and preincubated for 2h or 6h at 39℃. Ejaculated fresh semen for each replicate was collected from 2 different bulls using an artificial vagina. According to the method of Iritani

*et al.* (1984), semen samples were kept in a stoppered 10 ml test tube at 20℃ for 18h by centrifugation at 500×g for 10 min. The sperm pellets were resuspended with DM and pre-incubated for 6h at 39℃ in al $CO_2$ incubator.

### 3. *In vitro* fertilization(IVF)

DM or DM(−BSA) containing 10 or 15% FCS was used for IVF. Following preincubation of 2h or 6h, sperm suspension was diluted with IVF medium to adjust the final concentration of spermatozoa of $1\sim2\times10^6$ motile sperm per ml of IVF medium. Sperm and oocytes were incubated together in 0.4ml of media under paraffinoil for 36h at 39℃ in 5% $CO_2$ 95% air at high humidity. After co-incubation, the oocytes were mechanically freed form surrounding cells and were observed for the number of apparently cleaved oocytes.

## RESULTS AND DISCUSSION

### 1. *In vitro* maturation of follicular oocytes

Table 1 and 2 each shows that the *in vitro* maturation rates of oocytes cultured in DM (−BSA) and Ham's F10 containing 5 to 20%FCS, respectively. As shown in Tables, the proportions of oocytes reached metaphase Ⅱ were 51.1 to 64.7% in DM(−BSA) plus FCS and 57.1 to 70.0% in Ham's F10 plus FCS. Although there were no significant differences among the levels of FCS for maturation, these maturation rates were higher than those of DM (−BSA) or Ham's F10 only(50% and 40%), respectively, FCS improved the maturation rate of oocytes and more favorable effect of FCS on *in vitro* maturation could be obtained form the addition of 15% for DM(−BSA) and 15 to 20% for Ham's F10. On the other hand, when Ham's F10 containing FCS was used in comparison with DM(−BSA) containing FCS for the oocyte maturation, Ham's F10 was not significantly more efficient than DM(−BSA). In our study, FCS showed a similar rate to the report of Shea *et al.* (1976) and Leibfried and First (1979) and a slightly higher rate than that of

Table 1. Maturation rate of follicular oocytes cultured *in vitro* in DM 28h

| Culture medium | No. of oocytes | Stage of meiosis | | | | | |
|---|---|---|---|---|---|---|---|
| | | GV | Met Ⅰ | Ana Ⅰ | Tel Ⅰ | Met Ⅱ | %Met Ⅱ |
| Defined medium(DM) | 16 | 2 | 5 | | | 7 | 50.0 |
| DM(−BSA) + 5%FCS | 16 | | 3 | 3 | | 10 | 62.5 |
| DM(−BSA) +10%FCS | 14 | | 4 | 1 | | 9 | 64.3 |
| DM(−BSA) +15%FCS | 17 | | 3 | 2 | 1 | 11 | 64.7 |
| DM(−BSA) +20%FCS | 14 | | 6 | | | 8 | 57.1 |

Table 2. Maturation rate of follicular oocytes cultured *in vitro* in Ham's F10 for 28h

| Culture medium | No. of oocytes | Stage of meiosis | | | | | |
|---|---|---|---|---|---|---|---|
| | | GV | Met Ⅰ | Ana Ⅰ | Tel Ⅰ | Met Ⅱ | %Met Ⅱ |
| Ham's F10 | 16 | 2 | 5 | | | 7 | 50.0 |
| Ham's F10+ 5%FCS | 16 | | 3 | 3 | | 10 | 62.5 |
| Ham's F10+10%FCS | 14 | | 4 | 1 | | 9 | 64.3 |
| Ham's F10+15%FCS | 17 | | 3 | 2 | 1 | 11 | 64.7 |
| Ham's F10+20%FCS | 14 | | 6 | | | 8 | 57.1 |

Fukui *et al.* (1987). Numberous studies published concerning *in vitro* maturation have been added to maturation media with FCS as a protein supplement. In these cases, the oocytes were allowed to mature at FCS concentration of 15% (Shea *et al.*,1976) or 20%(Fukui *et al.*, 1987) in Ham's F10 or F12 medium and FCS concentration of 10%(Leibfried-Rutledge, *et al.*,1987; Niwa and Ohgoda, 1988;Sirard *et al.*,1988) and 20%(Fukui and Ono, 1989; Younis *et al.*, 1989) in TCM199 medium, respectively.

### 2. *In vitro* fertilization of *in vitro*-matured oocytes

Table 3 shows the cleavage rates(as a criterion of IVF) of oocytes matured in DM (−BSA) containing 50 to 20% FCS. The FCS addition to DM without BSA and its levels did not affected the cleavage rates. The use of unfrozen-diluted semen provided a higher cleavage rate compared to frozen-thawed semen.

Table 4 shows the cleavage rates of oocytes matured in Ham's F10 or TCM199 containing 10% FCS, respectively. These rates were higher than those of Table3. These was no difference in the cleavage rate between maturation media and there also was not different between sperm treatments. As shown in Table 3 and 4, the IVF media did not affect the cleavage rates. The present result that FCS did not improve the cleavage rates was not similar to those of other investigators. In the rats, Vanderhyden and Amstrong(1989) demonstrated that maximum normal fertilization obtained at FCS concentration of 15% in maturation medium and serum improved the fertilizability of oocytes compared with oocytes matured with BSA. Also, Schroeder and Eppig(1984) and Choi *et al.* (1987) in the mouse and Leibfried-Rutledge *et al.* (1986), Sanbuissho and Threfall(1989) and Kim *et al.* (1989) in the bovines have shown the importance of serum in maturation medium. Their results in the bovine oocytes indicated that serum provided a superior environment for oocyte maturation as compared with BSA. However, this beneficial effect of serum in supporting oocyte maturation and fertilization should be further studied for the success of IVF and the development of *in vitro*-matured oocytes, because the precise role fo serum and the effect of serum levels on maturation and IVF is not fully understood as well as although FCS is routinely used in culture medium(Wright *et al.*,1976; Camous *et al.*,1984;Fukui and Ono,1989).

In our study, the result that showed more higher cleavage rates from unfrozen semen was in contrast to those of Bondioli and Wright (1983) and Fukui *et al.* (1987), who reported that the type of sperm(frozen and unfrozen) did not significantly affect the IVF and cleavage rates of follicular oocytes. The higher cleavage rates from unfrozen semen was probably caused by the more complete capacitation of sperm as suggested by Iritani *et al.* (1984) and Sirard *et al.* (1985). On the other hand, the present result that the oocytes matured *in vitro* in Ham's F10 or TCM199 cleaved at equal rates but these rates were higher than those of oocytes matured in DM(−BSA) containing FCS was in agreement with the result of Ueda *et al.* (1988). However, the result was not similar to that of Sirard and Lambert(1985), who showed that DM was found to be superior to Ham's F10 for IVF and cleavage. Kuwayama *et al.* (1989) has suggested that TCM199 was superior to Ham's F10 for IVF and early cleavage. The cleavage rates with unfrozen-diluted semen in this study was similar to that reported by Sirard and Lamber (1985) and was higher than that of Hensleigh and Hunter(1985). The rates with frozen semen was similar to other reports(Fukui *et al.*, 1987;Kim *et al.*, 1988;Leibfried-Rutledge *et al.*,

Table 3. Cleavage rate of follicular oocytes matured *in vitro* in DM containing FCS for 28h

| Maturation medium | Sperm treatment | IVF medium | No. of oocytes (expanded) | Cleavel oocytes (2-3 cells) | Cleavage rate(%) |
|---|---|---|---|---|---|
| Defined medium | Frozen, HIS | DM | 9 | 1 | 11.1 |
| DM(−BSA)+ 5%FCS | 〃 〃 | 〃 | 14 | 0 | 0 |
| DM(−BSA)+10%FCS | 〃 〃 | 〃 | 11 | 1 | 0.9 |
| DM(−BSA)+15%FCS | 〃 〃 | 〃 | 12 | 0 | 0 |
| 〃 | Unfrozen, DM | DM(−BSA) +15%FCS | 6 | 2 | 33.3 |
| 〃 | 〃 HIS | 〃 | 5 | 2 | 40.0 |
| DM(−BSA)+20%FCS | Frozen, HIS | DM | 13 | 0 | 0 |

Duration of sperm preincubation was 6h in DM.

Table 4. Cleavage rate of follicular oocytes matured *in vitro* in Ham's F10 or TCM199 containing FCS for 28h

| Maturation medium | Sperm treatment (frozen semen) | IVF medium | No. of oocytes (expanded) | Cleavel oocytes (2-3 cells) | Cleavage rate(%) |
|---|---|---|---|---|---|
| Ham's F10+10%FCS | DM | DM | 7 | 1 | 14.3 |
| | 〃 | DM(−BSA) +10%FCS | 6 | 1 | 16.7 |
| | HIS | DM | 7 | 0 | 0 |
| | 〃 | DM(−BSA) +10%FCS | 10 | 1 | 10.0 |
| TCM199+10%FCS | DM | 〃 | 30 | 5 | 16.7 |
| | HIS | 〃 | 30 | 5 | 16.7 |

Duration of sperm preincubation was 2h in DM.

Table 5. Sperm acrosome reaction of frozen semen preincubated in DM after treatment or non-treatment of HIS

| Preinculation time(h) | HIS treatment(+) or not(−) | Acrosome reaction(%) |
|---|---|---|
| 0 | − | 3.8± 1.2 |
| 2 | − | 38.9± 4.9 |
| | + | 40.6± 2.3 |
| 4 | − | 53.5±13.4 |
| | + | 50.3± 2.6 |
| 6 | − | 53.5 ± 8.0 |
| | + | 63.0 ± 3.4 |

1987) but greatly lower than that of Kajihara *et al.* (1987). Table 5 shows the acrosome reaction of frozen-thawed sperm used to obtain the data of Table 3 and 4. The proportions of acrosome

reacted sperm after 2h-and 6h-preincubation was 39 and 54 for DM only and 41 and 63 for HIS treatment, respectively. These results indicated that the lower cleavage rates in Table 3 than in Table 4 might be partially related to low sperm motility due to the prolonged preincubation time for 6h after HIS since the percentage of AR was negatively correlated with sperm motility.

In conclusion, the results presented here show that relatively high maturation rates of follicular oocytes could be obtained in DM(−BSA) medium supplemented with FCS. However, the IVF of these oocytes was not significantly improved. Ham's F10 or TCM199 which is biochermically more complete than DM was thought to be more efficient for the oocyte maturation and the procedure of sperm treatment was also a important factor to improve the IVF or cleavage of oocytes matured *in vitro*.

## ACKNOWLEDGEMENTS

These studies were conducted under a grant (87 project) from the Ministry of Education of the Republic of Korea. The authors thank the Dairy and Korean Native Cattle Improvement Center and the Seoul Livestock Products Marketing Center, NLCF, for providing the bull semen and cow ovaries, respectively and also graduate students, who aided in these studies, of Department of Animal Science Chung-Ang University.

## SUMMARY

Several levels of fetal calf serum(FCS) were evaluated for their ability to enhance the *in vitro* maturation of bovine follicular oocytes and the cleavage rate of *in vitro*-fertilized oocytes. FCS was added at 5 to 20%(v/v) to the maturation media(Brackett's defined medium without BSA, Ham's F10 and TCM199). Oocytes were collected from 2~6 mm follicles, cultured for 28h in a $CO_2$ incubator at 39℃ and fertilized with frozen-thawed or unfrozen semen capacitated in vitro. The oocytes reached metaphase II was 57 to 65% in DM(−BSA) plus BSA and 57 to 70% in Ham's F10 plus FCS(control; 50 and 40%, respectively). These was no difference between two different media and among 4 levels of FCS. FCS addition to DM(−BSA) for maturation and its levels did not affect the cleavage rates of oocytes. The cleavage rate of IVF oocytes was greatly low by 6h-preincubated frozen semen after HIS treatment but was increased by 2h-preincubated frozen semen and significantly increased by the use of unfrozen semen capacitated *in vitro*. The cleavage rates of oocytes matured in Ham's F10 or TCM199 plus 10% in DM(−BSA) plus FCS. These results indicate that *in vitro* maturation of oocytes improved by the addition of FCS to medium but did not greatly affect the cleavage rates after IVF and that the cleavage rates varied by sperm conditions and FCS addition to Ham's F10 or TCM199 was more efficient for oocytes maturation.

## REFERENCES

Bondioli KR and Wright RW. 1983. *In vitro* fertilization of bovine oocytes by spermatozoa capactation *in vitro*. J. Anim. Sci. 57:1001-1005.

Brackett BG, Bousguet D, Boice ML, Donawick WJ, Evans JF and Dressel MA. 1982. Normal development following *in vitro* fertiliz ation on the cow. Biol. Reprod. 27:147-158.

Brackett BG and Oliphant G. 1975. Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12:260-274.

Camous S, Heyman Y, Meziou W and Menezo Y. 1984. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblatic vesicles. J. Repord. Fertil. 72:479-485.

Choi TS, Mori M, Kohmoto K and Shoda. Y. 1987. Beneficial effect of serum on the fertilizability of mouse oocytes matured *in vitro*. J. Reprod. Fertil. 79:565-568.

Fukui Y, Fukushima M and Ono H. 1984. Effects of various sperm procedures on fertilization of bovine follicular oocytes in rabbit reproductive tract after maturation *in vitro*. Jpn. J. Fertil. Steril. 29:117.

Fukui Y, Imai K, Alfonso N and Ono H. 1987. Follicle culture enhances fertilizability and cleavage of bovine oocytes matured *in vitro*. J. Anim. Sci. 64:935-941.

Fukui Y and Ono H. 1989. Effects of sera, hormones and granulosa cells added to culture medium for *in-vitro* maturation, fertilization, cleavage and development of bovine oocytes. J. Reprod. Fertil. 86:501-506.

Hensleigh HC and Hunter AG. 1985. *In vitro* maturation of bovine cumulus enclosed primary oocytes and their subsequent *in vitro* fertilization and cleavage. J. Dairy Sci. 68:1456-1462.

Iritani A. 1987. Recent advances in embryology and embryo manipulation and their potential contribution to agriculture. 4th AAAP Anim. Sci. Congr. Feb 1~6, Hamilton, NZ. pp. 126-129.

Iritani A, Kasai M, Niwa K and Song HB. 1984. Fertilization *in vitro* of cattle follicular oocytes with ejaculated spermatozoa capacitated in a chemically defined medium. J. Reprod. Fertil. 70:487-492.

Iritani A and Niwa K. 1977. Capacitation of bull spermatozoa and fertilization "*in vitro*" of cattle follicular oocytes matured in culture.

Kajihara Y, Goto K, Kosaka S, Nakanishi Y and Ogawa K. 1987. *In vitro* fertilization of bovine follicular oocytes and their development up to hatched blastocysts *in vitro*. Jpn. J. Anim. Reprod. 33:173-180.

Kim CK, Chung YC, Park JW and Song HB. 1988. Studies on maturation *in vitro* and fertilizing ability of bovine follicular oocytes. Korean J. Anim. Sci. 30:224-232.

Kim CI, Ellington JE and Foote RH. 1989. *In vitro* maturation and development of bovine oocytes. Theriogenology 31:210.

Kuwayama M, Shioya Y, Iwasaki S, Okuyama Y, Fukushima M and Hanada A. 1989. Effects of culture medium and time of transfer to the rabbit oviduct on the developmental capability fo bovine oocytes matured and fertilized *in vitro*. Jpn. J. Anim. Reprod. 35:1-6.

Leibfried-Rutledge ML, Critser ES and First NL. 1986. Effects of fetal calf serum and bovine serum albumin on *in vitro* maturration and fertilization of bovine and hamster cumulus-oocyte complexes. Biol. Repord. 34:850-857.

Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL and First NL. 1987. Developement potential of bovine oocytes matured *in vitro* or *in vivo*. Biol. Reprd. 36:376-383.

Leibfried ML and First NL. 1979. Characterization of bovine follicular oocytes and their ability to mature *in vitro*. J. Anim. Sci. 48:76-86.

Niwa K and Ohgoda O. 1988. Synergistic effect of caffeine and heparin on *in-vitro* fertiliz ation of cattle oocytes matured in culuture. Theriogenology 30:733-741.

Parrish JJ, Susko-Parrish JL, Leib-fried-Rutledge ML, Critser ES, Eyestone WH and First NL. 1986. Bovine *in vitro* fertilization

with frozen-thawed semen. Theriogenology 25:591-600.

Sanbuissho A and Threfall WR. 1989. The effect of estrous cow semen on the *in vitro* maturation and fertilization of the bovine follicular oocyte. Theriogenology 31:693-699.

Schroeder AC and Eppig JJ. 1984. The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. Dev. Biol. 102:493-497.

Shea BF, Latour JPA, Bedirian KN and Baker RD. 1976. Maturation *in vitro* and subsequent penetrability of bovine follicular oocytes. J. Anim. Sci. 43:809-815.

Sirard MA and Lambart RD. 1985. *In vitro* fertilization of bovine follicular oocytes by laparoscopy. Biol. Reprod. 33:487-494.

Sirard MA, Lambert RD, Menard DP and Bedoya M. 1985. Pregnancies after *in vitro* fertilization of cow follicular oocytes, their incubation in rabbit oviduct and their transfer to the cow uterus. J. Reprod. Fertil. 75:551-556.

Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML and First NL. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. Biol. Reprod. 39-546-552.

Ueda S, Kuwayama M, Shioya Y and Hanada A. 1988. Culture of *in vitro* fertilized bovine embryos in a chemically defined medium (BO-medium). Jpn. J. Anim. Reprod. 34:56-60.

Vanderhyden BC and Armstrong DT. 1989. Role of cumulus cells and serum on the *in vitro* maturation, fertilization, and subsequent development of rat oocyte. Bio. Reprod. 40:720-728.

Wright RW, Anderson GB, Cupps PT and Drost M. 1976. Successful culture *in vitro* of bovine embryos to the blastocyst stage. Biol. Reprod. 14:157-162.

Wright TE and Bondioli KR. 1981. Aspects of "*in vitro*" fertilization and embryo culture in domestic animals. J. Anim. Sci. 53:702-729.

Younis AI, Brackett BG and Fayer-Hosken RA. 1989. Influence of serum and hormones on bovine oocyte mauration and fertilization *in vitro*. Gamete Res. 3:3:189-201.