韓國家畜繁殖學會誌 14(1) : 84~91(1990)
Korean J. Anim. Reprod.

돼지 난포란의 체외성숙에 관한 연구

김창근 · 정영채 · 이명식 · 윤종택 · 방명걸* · 정길생**

중앙대학교 축산학과

Studies on *In Vitro* Maturation of Pig Follicular Oocytes

Kim, C.K, Y.C. Chung, M.S.Lee, J.T.Yoon, M.G. Pang*, and K. S. Chung**

Department of Animal Science, Chung-Ang University

적 요

본 실험은 돼지난포란의 체외성숙과 체외수정 효과를 높일 수 있는 방법을 찾기 위하여 시도되었으며 직경 1~2 mm와 3~7 mm 난포로부터 채란된 난자를 mKRB(-BSA)에 돼지발정혈청(ESS), FCS 또는 투석돼지 난포액(DFF)을 첨가한 성숙배양액에서 24~48시간, 37℃에서 배양하였다. 성숙된 난포란은 정소상체 정자와 24시간 배양 후 전핵형성 여부를 조사하였다. 36~48시간 배양에서 50~60%의 난자가 metaphase II에 도달되었고 난포 크기 (1~2 mm 와 3~7 mm)간에 체외성숙율의 차이는 없었으나 3~7 mm 난포란에서 성숙분열이 다소 빨랐다. 체외성숙배양액에 5% ESS, 15% FCS 및 DFF 첨가시 대조구보다 다소 성숙율이 높았다. 체외수정율(전핵형성)은 5% ESS 와 15% FCS 첨가 성숙시킨 난포란과 체내 수정능획득 정자와의 수정에서 각각 높은 경향이 있었다. 따라서 돼지난포란의 체외성숙과 수정에 ESS, FCS 및 투석난포액이 유효한 요인이 됨을 알 수 있었다.

(Key Words: estrous sow serum, *in vitro* fertilization, *in vitro* maturation, pig follicular oocytes, pronucleus formation)

I. INTRODUCTION

The development of efficient methods for *in vitro* maturation and fertilization of follicular oocytes has been requested for the further exploitation of nuclear transplantation and genetic manipulation in farm animals. In the pigs, the successful *in vivo* fertilization of *in vitro*-matured follicular oocytes was achieved by Motlik and Fulka(1974) and *in vitro* fertilization of such oocytes was first reported by Iritani et al.(1978). Recently, Mattioli et al.(1989) suggested that the pig oocytes matured and fertilized *in vitro* can establish normal pregnancy.

However, the development potenitial of *in vitro*-fertilized follicular oocytes was still very low. Initani et al.(1978) and Nagai et al.(1984)

本 研究는 1987年度 文敎部 遺傳工學 學術研究費에 의하여 수행되었음.

*서울대학교 산부인과 (Department of Obstetrics and Gynecology, Seoul National University)

**건국대학교 축산학과 (Department of Animal Science, Konkuk University)

indicated that these inferior development might be due to the poor male-pronucleus formation following the incomplete cytoplasmic maturation of oocytes. There remains a need for more detailed studies on culture conditions that will enable the high proportion of pig oocytes to undergo the *in vitro* maturation, pronucleus formation and cleavage(Motlik and Fulka, 1974 ; Naito et al., 1988 ; Mattioli et al., 1989).

To induce the *in vitro*-full maturation of follicular oocytes, serveral important factors have been considered. A correlation between the size of antral follicles and the frequency of pig oocytes completing *in vitro* maturation was reported(Tsafriri and Channing, 1975 ; McGaughey et al., 1979 ; Motlik et al., 1984). Naito et al.(1988, 1989) demonstrated that the male-pronucleus formation capacity and developmental ability of *in vitro*-matured pig oocytes improved markedly by the addition of follicular fluid to a maturation medium.

Recent studies in the bovine have shown that the supplement of serum provided a superior environment for *in vitro* oocyte maturation as compared with BSA(Sanbuissho and Threfall, 1985 ; Younis et al., 1989) and estrous serum tended to increase the cleavage rate and development into blastocyst as compared with FCS(Fukui, 1989). However, these favorable effects of serum on *in vitro* maturation and fertilization have been very limited in the pig oocytes.

This study was undertaken to investigate the ability of pig oocytes derived from different follicular size to undergo *in vitro* maturation, the effect of serum and dialyzed pig follicular fluid on *in vitro* maturation and the fertilization of *in vitro*-matured oocytes with epididymal spermatozoa.

II. MATERIALS AND METHODS

1. Collection of follicular oocytes

Crossbred gilt ovaries which had no corpus luteaum were obtained at a local abattoir and were maintained in a thermos containing saline solution(30~35℃) during transit to laboratory.

Within 2hr after slaughter, the contents of follicles classified into two different groups(1~2 mm and 3~7mm in diameter) were separately aspirated with a syringe and 20guage needle and the contents were pooled each in a conical 50ml centrifuge tube.

The collected oocytes were rinsed twice in a mKRB solution and only oocytes with tightly adherent cumulus cells were used for culture.

2. In vitro maturation of follicular oocytes

Maturation was accomplished in a mKRB solution(Toyoda and Chang, 1974) and mKRB solution plus fetal calf serum(FCS), estrous sow serum(ESS) or dialyzed pig follicular fluid (DFF) instead of bovine serum albumin(BSA), respectively. The follicular fluid collected from 3~7mm follicles was dialyzed in a cellulose dialysis bag(12,000 dalton cut off).

The oocytes were cultured in 0.4ml of medium in Falcon dishes for 24~48hr at 37℃ under an atmosphere of 5% CO_2-95% air with high humidity.

At the end of each culture period, the oocytes were fixed with acetic-alcohol(1 : 3), stained by 1% aceto-orcein, and examined by a phase-contrast microscope.

3. Preparation of epididymal spermatozoa

Boar epididymides were removed at an abattoir and epididymal spermatozoa were collected. Semen(0.5ml) was supended in 3ml mKRB solution and then washed twice by

centrifugation for 5 min at 350 × g to remove the epididymal plasma.

The washed spermatozoa were resuspended in a mKRB solution to give the concentration of $4\sim8\times10^8$ cells/ml and the 0.5 ml of spermatozoa suspension was preincubated *in vitro* in a CO_2 incubator or *in vivo* a ligated mouse uterus for 4 hr.

4. *In vitro* fertilization

About 0.01 ml spermatozoa suspension ($1\sim2\times10^6$ cells/ml) preincubated according to the above procedure were introduced into the 0.4 ml IVF medium containing 10~15 *in vitro*-cultured oocytes.

After incubation for 24 hr with preincubated spermatozoa, the oocytes were fixed, stained and examined regarding the fertilization based on pronuclei formation.

III. RESULTS

1. *In vitro* maturation of follicular oocytes

The maturation rates at different intervals of the oocytes obtained from 1~2 mm or 3~7 mm follicles are shown in Table 1.

There was no difference in the oocyte maturation between two follicular size groups, although the 3~7 mm follicular group showed a slightly higher maturation rates.

About 50~60% of the oocytes in both two groups reached metaphase II during 36 to 48 hr of culture and 60% of the 3~7 mm follicular oocytes reached metaphase I at 24 hr of culture. Table 2 and 3 show the additional effect of ESS or FCS and dialyzed follicular fluid to the mKRB solution without BSA on the maturation rates of 3~7 mm follicular oocytes, respectively.

As shown in Table 2, the addition of 5% ESS and 15% FCS showed a slightly higher rates than that of the control mKRB with BSA. In Table 3, the maturation rates were also slightly increased by the additions of dialyzed follicular fraction.

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

Table 4 shows the *in vitro* fertilization of *in vitro*-matured follicular oocytes inseminated with *in vivo*- and *in vitro*-capacitated spermatozoa. The fertilization rate was increased when

Table 1. Maturation *in vitro* of oocytes derived from follicles of 1~2 mm and 3~7 mm in diameter

Follicular size (mm)	Duration of culture (h)	No. of oocytes examined	Stage of meiosis								Meturation rate (Met II, %)
			GV	Pro I	Met I	Ana I	Tel I	Met II	Deg	Uncertain	
1~2	24	29	2	2	15	2		5		1	17.2
	26	26			7		3	13	3		50.0
	48	32			6		2	19	2	2	59.4
3~7	24	17			10	1		5			29.4
	36	33			9	2		15	2		45.5
	48	31		1	8		1	19	2		61.3

Maturation medium was a mKRB solution containing 1 mg BSA/ml.

Table 2. Effect of ESS or FCS on *in vitro* maturation of 3~7 mm follicular oocytes cultured *in vitro* for 30 hr.

Maturation medium*	No. of oocytes examined	Stage of meiosis						Maturation rate (Met II, %)
		GV	Met I	Ana I	Tel I	Met II	Deg	
mKRB + 1 mg BSA/ml	108	14	35	6	5	54	4	50.0
mKRB(-BSA) + 5% ESS	96	3	28	3	5	56	1	58.3
mKRB(-BSA) + 10% ESS	23	3	7	-	1	11	1	47.8
mKRB(-BSA) + 15% ESS	21	4	4	1	2	9	1	42.9
mKRB(-BSA) + 15% FCS	41	4	7	3	4	23	-	56.1

ESS : Estrous sow serum, FCS : Fetal calf serum.

Table 3. Maturation *in vitro* of follicular oocytes cultured for 30 hr in medium containing dialyzed medium size-follicular fluid (DFF)

Maturation medium*	No. of oocytes examined	Stage of meiosis						Maturation rate (Met II, %)
		GV	Met I	Ana I	Tel I	Met II	Deg	
mKRB + 1 mg BSA/ml(A)	37	2	12	1	1	19	2	51.4
A + 10% DFF	33	6	5	2	1	19	-	57.6
A + 20% DFF	32	2	6	-	3	20	1	62.5
A + 50% DFF	29	2	9	1	1	16	-	55.2
100% DFF	23	1	8	-	-	14	-	60.0

mKRB(-BSA) plus 5% ESS or 15% FCS was used for the oocyte maturation.

All the 3 media showed a slightly higher fertilization rate for the oocytes inseminated with *in vitro*-capacitated spermatozoa than for the oocytes inseminated with *in vitro*-capacitated spermatozoa.

IV. DISCUSSION

In the present study maturation rates within 24~48 hr of culture confirmed the previous findings of Koh et al. (1988) and Yoshida et al (1989). Yoshida et al. (1989) reported that the rate of oocytes reaching metaphase II was significantly higher after 30 hr of culture than after 24 hr and there was no difference in the maturation rates between 30 and 48 hr of culture in the control group and between 36 and 48 hr of culture in the gonadotropin-added group.

The time required for maturation to metaphase II is in accordance with the report of Sato et al. (1978) but was shorter than that quoted by of McGauhey and Polge (1971). In addition, the time in which a high proportion of oocytes reached metaphase I was similar to that of Byun et al. (1989) but faster than that of McGaughey and Polge (1971).

The results that we found no difference in the maturation rates between the oocytes from

Table 4. *In vitro* fertilization (pronucleus formation) of *in vitro*-matured oocytes inseminated with *in vivo*-and *in vitro*-capacitated epididymal spermatozoa

Maturation medium	Sperm capacitation	No. of oocytes*			No. of fertilized oocytes with pronucleus(PN)					
		Matured (%, A)	Inseminated (B)	(C)	1PN	2PN	>2Pn	Total (D)	D/B (%)	D/C (%)
mKRB + 1mg BSA/ml	*In vivo*	16/32 (50.0)	36	(18)	8	1	1	10	(27.8)	(55.8)
	In vitro	28/58 (48.3)	17	(8)	2	-	-	2	(11.8)	(25.0)
mKRB(-BSA) + 5% ESS	*In vivo*	9/17 (53.0)	25	(13)	3	3	-	6	(24.0)	(46.2)
	In vitro	27/48 (56.3)	71	(40)	11	4	1	17	(23.9)	(42.5)
mKRB(-BSA)	*In vivo*	14/25 (56.0)	16	(9)	7	-	-	7	(43.8)	(73.3)
	In vitro	9/16 (56.3)	20	(11)	2	2	2	4	(20.0)	(36.4)

*A : A part of cultured oocytes perior to insemination were randomly selected and they were stained to evaluate nuclear maturation.

B : The remaining cumulus-expanded oocytes were inseminated to evaluate *in vitro* fertilization (the ability to support male pronuclear formation).

C : No. of matured oocytes = No. of oocytes inseminated (B) × maturation rate (%, A) of random samples.

small and large follicles differed from other reports(Tsafriri and Channing, 1975 ; McGaughey et al., 1979 ; Motlik et al., 1984), who reported that the oocyte maturation increased with an increase in follicular sizes.

In contrast, Motlik et al.(1984) found that the ability to complete meiotic maturation acquired antral follicles of about 2mm in diameter and the substantial difference between small and large follicles appeared after 48hr of culture.

However, because of the *in vitro* maturation competence of small follicular pig oocytes has been shown the conflicting results(McGaughey et al., 1979; Anderson and Hillensjo, 1982), further studies using a larger number of oocytes are needed to confirm the effect of follicular size on *in vitro* maturation rates of oocytes.

The present study found that the maturation rates could be improved by the addition of serum, as observed in the bovine oocytes(Kim et al., 1988; Younis et al., 1989). The beneficial effect of follicular fluid was similar to the result observed in the pig (Naito et al., 1988, 1989) and the improved maturation rate by the addition of FCS agreed with the reports of Fukui et al.(1982) and Minato and Toyoda (1982a).

However, the fact that maturation rates were less improved byd the addition of 10~15% ESS did not agree with those of the bovine oocytes. In addition, Tsafriri and Channing(1975) demonstrated in the pig oocytes that the increasing the serum level to 50% was beneficial for the maturation of oocytes from medium -sized follicles.

On the other hand, it is not possible to determine whether the beneficial effect of dialyzed follicular in the present study was due to the removal of its inhibitory substances

(Tsafriri and Channing, 1975; Meinecke and Meinecke Tillman, 1981) and harmful substances (Cho and Lim, 1975), or due to its beneficial factors (Naito et al., 1988, 1989).

We suggest that the favorable effect of dialyzed follicular fluid on maturation rates should be reconfirmed by an *in vitro* fertilization system. The proportion of fertilized oocytes was similar to that of Iritani et al. (1978). It is possible that the higher pronucleus formation obtained by *in vivo*-capacitated spermatozoa was related to the degree of spermatozoa capacitation process as described by others (Lenz et al., 1982).

Our result that the presence of serum in the culture medium during *in vitro* maturation improved the pronucleus formation may be explained by the results in other animals. In the mouse, it was reported that the addition of serum to a medium can improve the sperm penetration (Minato and Toyoda, 1982b ; Choi et al., 1987) and the further development of fertilized oocytyes (Schroeder and Eppig, 1984).

Eppig and Schroeder (1986) suggested that the serum prevented the zona pellucida hardening during *in vitro* maturation of oocytes and enhanced the potential of oocytes for fertilization and development.

Several studies in the bovine have shown that the serum provided a superior environment for oocyte maturation when compared with BSA (Sanbuissho and Threfall, 1985; Kim et al., 1988; Younis et al., 1989).

In conclusion, the present study indicates that the addition of ESS and FCS at proper level to a culture medium would be necessary to improve the *in vitro* maturation and pronucleus formation of pig follicular oocytes and the results further suggest that dialyzed follicular fluid might be important for *in vitro* maturation.

V. SUMMARY

Experiments were disigned to define and optimize efficiency of a system whereby pig follicular oocytes could be matured and fertilized *in vitro*. The pig oocytes removed from 1~2 mm and 3~7 mm follicles were cultured *in vitro* in the mKRB(-BSA) solution containing estrous sow serum(ESS), FCS or dialyzed pig follicular fluid for 24 to 48 hr at 37℃.

The oocytes matured *in vitro* were evaluated after epididymal spermatozoa-oocyte incubation for 24 hr for pronucleus formation. 50~60% of the oocytes reached metaphase II during 36 to 48 hr of culture.

There was no differernce in oocyte maturation between two groups of follicular size but meiosis was slightly faster in the 3~7 mm follicular oocytes.

The oocytes matured in mKRB(-BSA) plus 5% ESS, 15% FCS or dialyzed follicular fraction showed slightly higher maturation rates than the control mKRB. *in vitro* fertilization, pronucleus formation, tended to be increased when mKRB(-BSA) plus 5% ESS or 15% FCS was used for oocyte maturation and *in vivo* -capacitated spermatozoa were inseminated, respectively.

It is concluded that ESS, FCS and dialyzed pig follicular fluid may be effective factors for *in vitro* maturation and fertilization of pig follicular oocytes.

VI. ACKNOWLEDGMENTS

This research work was supported by grants from Ministry of Education, the Republic of Korea (1987 Project) and by the Institute of Genetic Engineering, Chung-Ang University.

We are grateful for technical assistance of

laboratory group, Department of Animal Science, Chung-Ang University.

VII. REFERENCES

1. Anderson, L.D. and T. Hillensjo. 1982. Lack of effect of gonadotropin-releasing hormone agonist upon meiosis in isolated porcine oocytes. Acta Physiol. Scand. 114 : 623-625.
2. Byun, T.H., K.S.Shin and H.B.Song. 1989. The effects of culture media on the *in vitro* maturation of procine follicular oocytes. Korean J. Anim. Sci. 31 : 363-372.
3. Cho, W.K. and K.J. Lim. 1975. Studies on the effects of follicular fluid and its fractions on the cow oocyte maturation *in vitro*. Korean J. Zool. 18 : 61-70.
4. Choi, T.S., M. Mori, K. Kohmoto and Y. Shoda. 1987. Beneficial effect of serum on the fertilizability of mouse oocytes matured *in vitro*. J.Reprod. Fert. 79 : 565-568.
5. Eppig, J.J. and A.C.Schroeder. 1986. Culture systems for mammalian oocyte development : Progress and prospects. Theriogenology 25 : 97-106.
6. Fukui, Y.1989. Effects of sera and steroid hormones on development of bovine oocytes matured and fertilized *in vitro* and co-cultured with bovine oviduct epithelial cells. J. Anim. Sci. 67 : 1318-1323.
7. Fukui, Y., Y. Terawaki and H. Ono. 1982. Effects of gonadotropin on *in vitro* maturation of bovine follicular oocytes. Jpn J. Fert. Steril. 27 : 1979-1987.
8. Iritani, A., K. Niwa and H. Imai. 1978. Sperm penetration of pig follicular oocytes matured in culture. J. Reprod. Fert. 54 : 379-383.
9. Kim, C.K., Y.C. Chung, J.W. Park and H.B. Song. 1988. Studies on maturation *in vitro* and fertilizing ability of bovine follicular oocytes. Korean J. Anim. Sci. 30 : 224-232.
10. Koh, T.H., K.S.Shin, T.H.Byun and J.K.Lee. 1988. *in vitro* maturation of porcine follicular oocytes. Korean J. Anim. Sci. 30 : 144-150.
11. Lenz, R.W., R.L. Ax, H.J. Grimek and N.L. First. 1982. Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. Biochem. Biophys. Res. Comm. 106 : 1092-1098.
12. Mattioli, M., M.L. Bacci. G. Galeati and E. Seren. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31 : 1201-1207.
13. McGaughey, R.W., D.H. Montgomery and J.D. Richter. 1979. Germinal vesicle configurations and patterns of polypeptide synthesis of porcine oocytes from antral follicles of different size, as related to their competency for spontaneous maturation. J. Exp. Zool. 209 : 239-254.
14. McGaughey, R.W. and C. Polge. 1971. Cytogenetic analysis of pig oocytes matured *in vitro*. J. Exp. Zool. 176 : 383-396.
15. Meinecke, B. and S. Meinecke-Tillman. 1981. Induction and inhibition of meiotic maturation of follicle-enclosed porcine oocytes *in vitro*. Theriogenology 15 : 581-589.
16. Minato, Y. and Y. Toyoda. 1982a. Effect of homologous serum and follicular fluid on the cumulus expansion and maturation division of porcine oocyte-cumulus complexes *in vitro*. Jpn J. Zootech. Sci. 53 : 515-520.
17. Minato, Y. and Y.Toyoda. 1982b. Fertilizing ability of mouse oocytes matured *in vitro*. Jpn J. Anim. Reprod. 28 : 134-140.
18. Motlik, J. and J. Fulka. 1974. Fertilization of pig follicular oocytes cultured *in*

vitro. J. Reprod. Fert. 36 : 235-237

19. Motlik, J., C. Nicole and J. Fulka. 1984. Meiotic competence *in vitro* of pig oocytes isiolated from early follicles. J. Reprod. Fert. 72 : 323-328.
20. Nagai, T., K. Niwa and A. Iritani. 1984. Effect of sperm concentration during preincubation in a defined medium on fertilization *in vitro* of pig follicular oocytes. J. Reprod. Fert. 70 : 271-275.
21. Naito, K., Y. Fukuda and I. Ishibashi. 1989. Developmental ability of porcine ova matured in porcine follicular fluid *in vitro* and fertilized *in vitro*. Theriogenology 31 : 1049-1057.
22. Naito, K., Y.Fukuda and Y.Toyoda. 1988. Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured *in vitro*. Gamete Res. 21 : 289-295.
23. Sanbuissho, A. and W.R. Threfall. 1985. The influence of serum and gonadotropins on bovine oocyte maturation *in vitro*. Theriogenology 29 : 301.
24. Sato, E., A. Iritani and Y. Nishikawa. 1978. Rate of maturation division of pig follicular oocytes cultured *in vitro*. Jap J. Zootech. Sci. 49 : 400-405.
25. Schroeder, A.C. and J.J. Eppig. 1984. The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. Dev. Biol. 102 : 493-497.
26. Toyoda, Y. and M.C.Chang. 1974. Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of such eggs following transfer, J. Reprod. Fert. 36 : 9-22.
27. Tsafriri, A. and C.P. Channing. 1975. Influence of follicular maturation and culture conditions on the meiosis of pig oocytes *in vitro*. J. Reprod. Fert. 43 : 149-152.
28. Yoshida, M., K. Bamba and Y. Kojima. 1989. Effects of gonadotropins and estradiol -17β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured *in vitro*. Jpn J. Anim. Reprod. 35 : 86-91.
29. Younis, A.I., G.B. Brackett and R.A. Fayer-Hosken. 1989. Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro*. Gamete Res. 23 : 189-201.