韓國家畜繁殖學會誌 23(3) : 239~245(1999)
Korean J. Animal Reprod.

Effects of Catalase and Cumulus Cells during *In Vitro* Maturation in Porcine Oocytes

Lee, S. Y.[1], S. J. Sa, K. J. Kim[2], H. T. Cheong, B. K. Yang, C. K. Park* and C. I. Kim
College of Animal Resources Science, Kangwon National University

돼지난자의 체외성숙시 Catalase와 난구세포의 영향

이상영[1] · 사수진 · 김광재[2] · 정회태 · 양부근 · 박춘근* · 김정익
강원대학교 동물자원과학대학

ABSTRACT

The effect of catalase on in-vitro maturation in porcine oocytes with or without cumulus cells were studied. The maturation rates were not significantly different in medium with and without catalase during *in vitro* maturation of oocytes. However, the maturation rates of oocytes with cumulus cells were significantly higher ($P<0.05$) than in oocytes without cumulus cells regardless of the presence of catalase. On the other hand, the maturation rate in oocytes cultured with cumulus cells for 48 h (57%) was significantly ($P<0.05$) higher than in oocytes with cumulus cells for first 24 h period (42%) only. In another experiment, the maturation rate was significantly ($P<0.05$) higher in medium containing catalase for last 24 h period only than in medium containing catalase for first 24 h period during *in vitro* maturation of oocytes with or without cumulus cells. But the oocytes matured to M-II stage were observed at 24 h of culture of oocytes without cumulus cells only. When oocytes with cumulus cells were cultured for 72 h, the maturation rates was significantly ($P<0.05$) higher in medium with (79%) than without (65%) catalase during *in vitro* maturation. These results indicate that cumulus cells are necessary for *in vitro* maturation of porcine oocytes, catalase have effect according to the addition periods and can prevent aging of porcine oocytes during maturation *in vitro*.

(Key words : *In vitro* maturation, Catalase, Cumulus cells, Porcine)

I. INTRODUCTION

In most mammalian species, oocytes are formed during fetal life and are arrested at the prophase stage of the first meiotic division until around the time of ovulation. Resumption of meiosis *in vivo* requires hormonal stimulation, which leads to GVBD and chromosomal condensation, followed by progression through metaphase of the first meiosis (MI), release of the first polar body, and then arrest at metaphase of

The author thanks B.C. Choi, S.T. Kim and D.W. Joo, Kangwon Province Veterinary Service Laboratory, for their assistance in collection of ovary.

[1] 낙농진흥회(Korea Dairy Committee)

[2] 강원도 가축위생시험소(Kangwon Province Veterinary Service Laboratory)

the second meiosis(M-II). The follicular wall of hemisectioned follicles also prevents GVBD in cumulus-enclosed oocytes grafted to the membrana granulosa (Meinecke and Meinecke-Tillmann, 1981). Porcine cumulus-enclosed oocytes cultured on follicular wall from which membrana granulosa cells were scraped off resume meiosis.

All porcine oocyte cumulus complexes isolated with a piece of membrana granulosa remain in the GV stage (Motlik et al., 1991). Data obtained *in vitro* with suspension of porcine granulosa cells are conflicting (Sirard and Bilodeau, 1990). Immature porcine oocytes with cumulus cells can mature spontaneously in suitable condition *in vitro*. Nevertheless, some investigators achived promising results related to in-vitro maturation culture (Fukui et al., 1991).

The gaseous environment is another important component of a culture system, and commonly, 5% CO_2 with 95% humidified air is used (Liu et al., 1995). However, the O_2 concentration of the oviduct is about one-third that of the atmosphere (Fischer and Bavister, 1993). Embryos cultured in high oxygen tension may produce more free radicals which are detrimental to embryo development. Reducing oxygen tention to 5 to 8% increase blastocyst development (Li and Foote , 1993). Free radicals can be degraded by enzymes such as superoxide dismutase, catalase and taurine. The presence of superoxide, taurine and catalase, which serve as radical scavengers in culture medium, have been found to have beneficial effects on embryonic development *in vitro* for bovine (Liu et al., 1995), and superoxide dismutase was found to be beneficial during *in vitro* maturation and fertilization of porcine oocytes (Park et al., 1996, 1997). Although correlations have been reported between the effectiveness of catalase and oocyte maturation *in vitro*, the relationship between catalase and cumulus cells during *in-vitro* maturation has not been elucidated.

The objectives of this study were to evaluate the effects and the role of catalase during *in-vitro* maturation in porcine oocytes with or without cumulus cells.

Ⅱ. MATERIALS AND METHODS

1. Oocyte Preparation

Porcine ovaries were collected at a local slaughterhouse and kept in saline (NaCl, 0.9% W/V ; penicillin 100,000 IU/l ; streptomycin 100mg /l and amphotericin B 250μg/l ; Sigma) at 30 to 32℃. Cumulus-oocyte complexes were aspirated from 2 to 6 mm follicles with a 10ml syringe with 18-G needle. The collected oocytes were washed three time in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium, 10 oocytes with a compact and complete cumulus cells were introduced to droplets (50μl) of maturation medium, and covered with mineral oil and cultured under the atmosphere of 5% CO_2 in air at 39℃.

2. Maturation Medium

The maturation medium consisted of TCM-199 with Earle's salt (Gibco, Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes, 10% FCS, 0.2 mM Na-pyruvate, 50 μg/ml gentamycin, 1 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol 17β and 10% (v/v) porcine follicular fluid (PFF).

3. Experimental Design

Experiment 1 : Oocytes were placed in maturation medium containing 0.1% (w/v) hyaluronidase and freed from cumulus cells by repeated passage through a fine pipette. Oocytes with or without cumulus cells were cultured in medium with (0.7mg/ml) or without catalase under 5% CO_2 in air at 39℃ for 48 h. During the

culture, hormones removed from maturation medium for second 24 h period.

Experiment 2 : Oocytes with or without cumulus cells were cultured in medium containing catalase for 48 h under culture conditions described Experiment 1. In another experimental group, oocytes freed from cumulus cells at 24 h of culture were cultured further 24 h in medium with catalase.

Experiment 3 : Effect of addition period of catalase during *in vitro* maturation of oocytes with or without cumulus cells was examined. The oocytes with or without cumulus cells were cultured in medium containing catalase for first 24 h period or second 24 h period.

Experiment 4 : Effect of catalase during in-vitro maturation with various periods were examined. The oocytes with or without cumulus cells were cultured for 24, 48 and 72 h.

4. Assessment of Oocyte Maturation

At various times after maturation, oocytes were mounted, fixed for 48~72 hrs in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope at magnification of ×200 and ×400. The stages were classified as germinal vesicle (GV), prophase I (P-I), metaphase I (M-I), anaphase I (A-I), telophase I (T-I), and metaphase II (M-II).

Ⅲ. RESULTS

In Experiment 1, when oocytes with cumulus cells were cultured for in-vitro maturation, the proportions of oocytes matured to M-II were not significantly different in medium with (67%) and without catalase(64%). There was also not significantly different in medium with(51%) and without(51%) catalase during in-vitro maturation of oocytes without cumulus. However, the maturation rates of oocytes with cumulus cells were significantly higher ($P<0.05$) than in oocytes without cumulus cells regardless of presence of catalase (Fig. 1).

In Experiment 2, the effect of durations of cumulus cells intacted during *in vitro* maturation in medium with catalase were examined. As shown Table 1, the maturation rate in oocytes with cumulus cells for 48 h (57%) was significantly ($P<0.05$) higher than in oocytes with cumulus cells for first 24 h period (42%) only during *in-vitro* maturation. However, significantly ($P<0.05$) lower maturation rate (30%) was obtained in culture with oocytes freed from cumulus cells for 48 h than in oocytes with cumulus cells for frist 24 h period or 48 h.

In Experiment 3, the effect of addition periods of catalase during *in vitro* maturation of oocytes with or without cumulus cells was examined. As shown Table 2, the proportion of oocytes matured was significantly ($P<0.05$) higher in medium containing catalase for last 24 h period (67%) only than in medium containing catalase for first 24 h period (48%) during *in vitro*

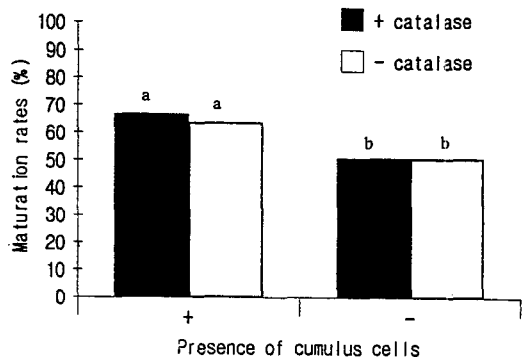

Fig. 1. Effect of catalase on *in vitro* maturation in porcine oocytes with or without cumulus cells. a-b Bars with different letters are significantly different ($P<0.05$).

Table 1. Effect of duration of cumulus cells intacted during in vitro maturation in medium with catalase in porcine oocytes

Presence of cumulus cells during culture		No. of oocytes examined	No. of oocytes matured		
0~24	24~48 (h)		GV	P-I~T-I	M-II (%)*
+	+	125	4	50	71(57)[a]
+	−	137	5	75	57(42)[b]
−	−	128	9	80	39(30)[c]

* GV:germinal vesicle, P-I :prophase-I, T-I :telophase-I, M-II :metaphase-II

[abc] Values with different superscripts in each column are significantly different ($P<0.05$).

Table 2. Effect of duration added of catalase during in vitro maturation of oocytes with or without cumulus cells

Presence of cumulus cells	Periods of catalase added during culture		No. of oocytes examined	No. of oocytes matured		
	0~24	24~48 (h)		GV	P-I~T-I	M-II (%)*
+	+	−	143	7	68	68(48)[a]
	−	+	129	4	40	85(67)[b]
−	+	−	132	7	69	56(42)[a]
	−	+	123	10	69	44(36)[a]

* GV:germinal vesicle, P-I :prophase-I, T-I :telophase-I, M-II :metaphase-II

[ab] Values with different superscripts in each column are significantly different ($P<0.05$).

maturation of oocytes with cumulus cells. In oocytes without cumulus cells, there was also higher in medium containing catalase for first 24 h period (42%) than for last 24 h period (36%) during *in vitro* maturation. However, the maturation rates were not significantly different in these two groups.

In Experiment 4, the relationship between cumulus cells and catalase for various periods of oocyte maturation *in vitro* were examined (Fig. 2). At 24 h after culture, the oocytes matured to M-II stage were observed in medium with (3%) or without (4%) catalase during *in vitro* maturation of oocytes without cumulus cells only. However, the maturation rates at 48 h of culture were significantly ($P<0.05$) higher in oocytes with (63 and 63%) than in oocytes without (50 and 51%) cumulus cells regardness of presence of catalase during *in vitro* maturation. On the other hand, when oocytes with cumulus cells were cultured for 72 h, the maturation rates was significantly ($P<0.05$) higher in medium with (79%) than without (65%) catalase during *in vitro* maturation. However, there were not significantly different in medium with (58%) and without (60%) catalase during *in vitro* maturation of oocytes without cumulus cells.

Ⅳ. DISCUSSION

The results of this study demonstrated that catalase are not influence nuclear maturation during *in vitro* maturation of porcine oocytes. However, more cultured oocytes (72 h) increased ability of porcine oocytes on *in vitro* maturation, and cumulus cells are also necessary for nuclear maturation. Resumption of GVBD in meiotically arrested oocytes was promoted fol-

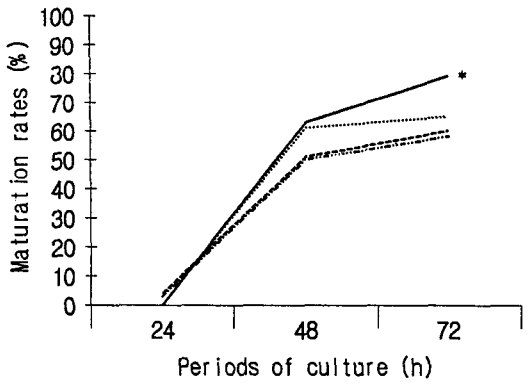

Fig. 2. Effects of cumulus cells and catalase for various periods of *in vitro* maturation in porcine oocytes. Porcine oocytes were maturated with cumulus cells and catalase(—), cumulus cells and without catalase(···), without cumulus cells and catalase(---) and without cumulus cells and catalase(-·-·). *Significantly different from the oocytes cultured in another groups for 72 h ($P<0.05$).

lowing pharmacological or growth factor(s) or by the addition of substrate. After liberation of porcine membrana granulosa layer from the follicular wall, the membrana granulosa has a strong tendency to roll up, but in an opposite way than in a follicle. This is the reason why complexes-oocytes cumulus were added upon the concave surface of basement membrane facing intrafollicularly to the theca layer (Kalous et al., 1993). Chian et al. (1994) reported that there were no significantly differences between oocytes cultured with or without cumulus cells during *in vitro* maturation in bovine. The pig membrana granulosa from prepubertal and PMSG-stimulated gilts prevented effectively resumption of meiosis in cattle cumulus-enclosed oocytes. Similarly, the porcine oocyte cumulus complexes isolated with a piece of membrana granulosa do not resume meiosis under *in vitro* conditions (Motlik et al., 1991). In the present study, the cumulus cells were required for in-vitro maturation of porcine oocytes regardness of presence of catalase. It was also required presence of cumulus cells continuously during *in vitro* maturation (Table 1).

In contrast to the numerous studies on the effects of catalase on male reproductive system, there are much less reports on the actions and role of catalase on oocyte maturation. The results of this study show that supplementation of catalase for *in vitro* maturation affects not ability of GVBD in porcine oocytes with or without cumulus cells (Fig. 1). In mammals, all cells are exposed to the risk of injury by active O_2 species, such as superoxide anion(O_2-) and hydrogen peroxide (H_2O_2), that are formed when molecular O_2 is utilized as an electron acceptor during oxidoreductive reactions in cells. These free radicals damage cell membranes, proteins, and nucleic acid (Freeman and Crapo, 1982). Park et al. (1997) reported that fertilization medium contains superoxide dismutase have a efficience for pronucleus formation in porcine oocytes. The limited capacity of male pronucleus formation in porcine oocytes matured and penetrated *in vitro* has been described by several investigators (Wang et al., 1991 ; Zheng and Sirard, 1992).

In the present study, there were not different by addition of catalase during *in vitro* maturation for 24 or 48 h in porcine oocytes. Taking these results into consideration, it is suggested that oocyte maturation is not protected from the oxidative stress under conditions with catalase in the maturation medium. Whereas early embryos are very sensitive to the toxic effects of reactive oxygen species, more mature embryos, especially after the implantation, are more resistant to reactive oxygen species. Tokura et al. (1992) reported that the superoxide anion is probably needed for their development since ad-

dition of superoxide dismutase to the incubation medium caused an important decrease in the formation rates of two germ cell layer and egg cylinder. In rabbit, concentrations of catalase ranging from 250 to 1000 IU did not affect the proportion of zygotes developing into blastocysts or the cell number. Therefore, any intra-embryonic accumulation of H_2O_2 would require an endogenous mechanism of removal, and added catalase would not be effected. In the present study, however, it was effected in medium added of catalase for last 24 h period than first 24 h period in oocytes with cumulus cells. This means that catalase addition is required for last 24 h period only during *in vitro* maturation of porcine oocytes.

In this study, porcine oocytes are incubated basically for about 48 h to maturation *in vitro*. At 24 h after culture, oocytes matured to M-II stage were obtained in oocytes without cumulus cells only (3 and 4 %, Fig. 2). However, the proportions of oocytes maturated at 48 or 72 h after culture were higher in oocytes with than without cumulus cells. It is possible that culture medium can promote in-vitro maturation of oocytes without cumulus cells for short periods of culture. In generally, it can suppose that oocytes matured for 72 h may be induce aging of porcine oocytes. In the present study, however, oocytes matured at 72 h of culture was significantly highest in oocytes culture with cumulus cells in medium containing catalase, and not different morphologically between oocytes cultured for 48 and 72 h. Thus, according to the present data, the catalase can prevent oocyte aging during *in vitro* maturation of porcine oocytes with cumulus cells.

In summary, the present study indicate that cumulus cells are necessary for *in vitro* maturation of porcine oocytes, the catalase can induce oocyte maturation by addition for last 24 h period during *in vitro* maturation. The facts that the maturation rates of normal oocytes were increased at 72 h after maturation in medium containing catalase suggests that catalase can prevent oocyte aging morphologically during oocyte maturation *in vitro*.

V. 요 약

본 연구는 돼지난자의 체외성숙시 난구세포와 catalase의 영향을 검토하고저 수행되었다. 체외성숙율은 catalase의 첨가유무에 관계없이 유의적인 차이는 인정되지 않았으나, 난구세포가 부착된 난자가 제거된 난자에 비해 유의적으로 높은 성숙율을 나타냈다($P<0.05$). 또한 48시간(57%)동안 난구세포가 부착된 경우 단지 처음 24시간(42%) 동안만 난구세포가 부착된 경우에 비하여 유의적으로 높은 성숙율을 나타냈다($P<0.05$). 한편, 난구세포가 부착 또는 제거된 난자의 성숙시 처음 24시간 동안 보다는 후반기 24시간 동안 catalase를 첨가한 경우 유의적으로 높은 성숙율을 나타냈으며($P<0.05$), 난구세포를 제거한 경우 성숙배양 24시간에서 M-II기로 성숙된 난자가 관찰되었다. 그러나 난구세포가 부착된 난자를 72시간 성숙배양 했을 때, catalase무첨가(65%) 보다는 첨가시(79%) 유의적으로 높은 성숙율을 나타냈다. 본 연구의 결과로부터 난구세포는 돼지난자의 체외성숙시 필수적인 것으로 인정되며, catalase는 첨가시기에 따라 난자의 성숙에 효과적으로 작용하였으며, 체외성숙시 난자의 노화를 방지할 수 가능성이 있는 것으로 추측된다.

VI. REFERENCES

1. Chian, R. C., K. Niwa and M. A. Sirard. 1994. Effects of cumulus cells on male pronuclear formation and subsequent early development of bovine oocytes *in vitro*. Theriogenology 41:1499-1508.
2. Fischer, B. and B. D. Bavister. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod.

Fertil. 99:673-679.

3. Freeman, B. A. and J. D. Crapo. 1982. Free radicals and tissue injury. Lab. Invest. 47: 412-426.
4. Fukui, Y., L. T., McGowan, R. W., James, P. A. Pugh and H. R. Tervit. 1991. Factors affecting the *in-vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. J. Reprod. Fertil. 92:125-131.
5. Kalous. J., P., Sutovsky. Z., Rimkeyicova, Y., Shioya, B. L. Lie and J. Motlik. 1993. Pig membrana granulosa cells prevent resumption of meiosis in cattle oocytes. Mol. Reprod. Dev. 34:58-64.
6. Li, J. and R. H. Foote. 1993. Culture of rabbit zygotes into blastocysts in protein-free medium with one to twenty percent oxygen. J. Reprod. Fertil. 98:163-167.
7. Liu, Z., R. H. Foot and X. Yang. 1995. Development of early bovine embryos in co-culture with KSOM and taurine, superoxide dismutase or insulin. Theriogenology 44:741-750.
8. Meinecke, B. and S. Meinecke-Tillman. 1981. Induction and inhibition of meiotic maturation of follicle- enclosed porcine oocytes *in vitro*. Theriogenology 15:581-589.
9. Motlik, J., T. Nagai and K. Kikuchi. 1991. Resumption of meiosis in pig oocytes cultured with cumulus and parietal granulosa cells: the effect of protein synthesis inhibition. J. Experi. Zool. 259:386-391.
10. Park, C. K., F. Roy and M. A. Sirard. 1996. The effect of free radicals and anti-oxidant during *in vitro* maturation and fertilization of porcine oocytes. Theriogenology 45:275 (abstr.).
11. Park, C. K., J. H., Lee, H. T., Cheong, B. K. Yang and C. I. Kim. 1997. Effect of superoxide dismutase on pronucleus formation of porcine oocytes fertilized *in vitro*. Theriogenology 48:1137-1146.
12. Sirard, M. A. and S. Bilodeau. 1990. Granulosa cells inhibit the resumption of meiosis in bovine oocytes *in vitro*. Biol. Reprod. 43:777-783.
13. Tokura, T., Y., Noda, K., Narimoto, Y., Umaoka, T. Mori and Ogawa, K. 1992. Effect of superoxide dismutase on the development following the implantation stage in mice. Acta Histochem. Cytochem. 25:491-498.
14. Wang, W. H., K. Niwa and K. Okuda. 1991. *In-vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. J. Reprod. Fertil. 93:491-496.
15. Zheng, Y. S. and M. A. Sirard. 1992. The effect of sera, bovine serum albumin and follicular cells on *in vitro* maturation and fertilization of porcine oocytes. Theriogenology 37:779-790.

(접수일자 : 1999. 7. 31. /채택일자 : 1999. 9. 20.)