Effect of Co-Culture with Mammalian Spermatozoa on *In Vitro* Maturation of Porcine Cumulus-Enclosed Germinal Vesicle Oocytes

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ABSTRACT

In vitro **maturation of denuded porcine immature oocytes can be enhanced by co-incubation with spermatozoa even before fertilization. This study was to determine whether the addition of spermatozoa into the culture medium could influence the nuclear maturation of porcine cumulus-enclosed germinal vesicle (GV) oocytes. Cumulus-oocyte complexes (COCs) were collected from follicles of 3- to 5-mm diameter. Porcine COCs were cultured in tissue culture medium containing spermatozoa. After 48 h culture, oocytes were examined for evidence of GV breakdown, metaphase I, anaphase-telophase I, and metaphase II. The proportion of oocytes reaching at metaphase II was significantly ($P<0.05$) increased in the oocytes cultured in media containing spermatozoa compared to those in media without spermatozoa (52.3% vs 12.5%). No difference in the percentage of metaphase II was observed among the different periods of spermatozoa exposure and among the spermatozoa from different species. The proportion of oocytes reaching metaphase II was significantly different between high and low concentrations of spermatozoa. The present study suggests that mammalian spermatozoa contain a substance(s) that improves nuclear *in vitro* maturation of porcine cumulus-enclosed GV oocytes. Enhancing effect of spermatozoa for *in vitro* maturation of oocytes is a highly dose-dependent.**

(Key words : *In vitro* maturation, Oocytes, Porcine, Spermatozoa)

INTRODUCTION

Oocytes maturation, characterized by germinal vesicle breakdown, formation of the first meiotic spindle (metaphase I), expulsion of the first polar body and the arrest in metaphase of second meiotic division (metaphase II), occurs in preovulatory follicles in response to the surge of gonadotropin and leads to an ovulated oocyte. However, in the more than 60 years since Pincus and Enzmann (1935) observed the spontaneous resumption of meiosis in mammalian oocytes released from follicular environment, oocytes nuclear maturation *in vitro* has been intensively studied. In rodents, spontaneous oocyte maturation is achieved in >95% of oocytes removed from their follicular environment (Vanderhyden and Armstrong, 1990). In humans, however, spontaneous maturation *in vitro* is achieved in only 30% to 50% of oocytes (Edwards, 1965; Cha et al, 1991). Protein and hormonal supplements are two principal factors known to influence the maturation process *in vitro* within various mammalian species. Maturation media are generally supplemented with protein, such as fetal calf serum (FCS) and BSA (Zheng and Sirard, 1992). Hormonal supplement are achieved by addition of various combination of FSH, LH and estradiol. Bovine (Goto et al, 1988) and rabbit (Yoshimura et al, 1989) oocytes matured in the absence of gonadotropins have, in fact, shown satisfactory developmental competence.

Gonadotropins, added *in vitro* to maturation media, enhance oocyte quality as shown by improved completion of nuclear maturation, fertilizability, and developmental ability (Wang and Niwa, 1995; Kaplan et al, 1978). Porcine oocytes matured in a medium supplemented with protein and estradiol but without FSH *in vitro* have poor ability to undergo germinal vesicle breakdown and to mature metaphase II (Nagai et al, 2000). Although it is generally accepted that cumulus cells during oocytes maturation support nuclear and cytoplasmic maturation for developmental competence after fertilization, *in vitro* maturation of porcine denuded GV oocytes could be significantly stimulated by co-incubation of spermatozoa without the supplementation of gonadotropin and protein (Kim et al, 2003).

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However, no data on addition of spermatozoa during *in vitro* maturation of porcine COCs has been published. For a better understanding of spermatozoa's enhancing effect, it is necessary to investigate the effect of co-culture with spermatozoa during *in vitro* maturation of mammalian COCs. Therefore, the present study was performed to determine whether *in vitro* nuclear maturation of porcine COCs could be enhanced by co-culture with spermatozoa in a hormone-free, chemically defined medium.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), unless stated otherwise.

Media

The medium used for maturation of oocytes was tissue culture medium (TCM) 199 with HEPES and supplemented with 100 IU/mL penicillin G, and 100 ìg/mL streptomycin sulfate (pH 7.3).

In Vitro Maturation

Porcine ovaries from random breeds were collected immediately post mortem at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% NaCl solution at 30 to 35℃. The ovaries were pooled regardless of the stage of the donors' cycle. COCs from follicles of 3 to 5 mm diameter were selected on the basis of their translucent appearance, good vascularization and the compact of granulosa layer and cumulus mass. COCs were washed four times in maturation medium. A group of 10 to 15 cumulus-enclosed oocytes were transferred into a 100 μL maturation medium under warm paraffin oil in a polystyrene culture dish, which has been previously kept for about 4 h in a CO_2 incubator. Oocytes were cultured at 39℃ under an atmosphere of 5% CO_2 and 95% air with high humidity.

Sperm Preparation

Cryopreserved ejaculated boar and bovine semen obtained from Kangwon University were used. Human semen was obtained from volunteers. Semen was collected by masturbation. In case where sperm showed any indication of movement, a sample was frozen. Sperm were washed in TCM 199 by centrifugation. Sperm pellets were resuspended in the same medium. An equal volume of cryopreservation medium was added to the sperm. The cryopreservation medium consisted of solution A (5.5 g NaCl, 0.4 g KCl, 0.03 g NaH_2PO_4, 2.4 g Tris, 0.1 g $MgSO_4$, 2.6 g $NaHCO_3$, 16 g sucrose, 0.5 g sodium citrate, 0.05 g sodium pyruvate, 0.7 g calcium lactate, and 10 g glycine per liter), egg yolkand glycerol. Freezing protocol started at room temperature, then cooled at a rate of 1.0℃/min to -70℃ and plunged into liquid nitrogen. The cryopreserved semen were thawed at 37℃ for about 1 min. Cryopreserved semen washed three times by centrifugation, each time at 1,200 × g for 1 min and re-suspended in TCM to give a concentration of 5 to 6 × 10^6 spermatozoa /mL.

Assessment of Oocytes

After 48 h culture, oocytes were mounted, fixed for 48 to 72 h in 25% acetic acid in alcohol (v:v) at room temperature, stained with 1% (v:v) orcein in 45% (v:v) acetic acid, and examined for evidence of germinal vesicle breakdown (GVBD), metaphase I, anaphase-telophase I and metaphase II.

Experimental Studies

In experiment 1, in order to examine the effect of co-culture of spermatozoa during *in vitro* maturation in porcine cumulus-enclosed oocytes, COCs were introduced into 100 μL of TCM 199 supplemented with 2.5 to 3× 10^6 spermatozoa/mL or TCM 199 alone (control).

In experiment 2, the effect of concentration of spermatozoa on *in vitro* maturation was examined. Concentrations of spermatozoa in the TCM 199 medium for oocytes maturation were adjusted to 2.5 to 3 × 10^3, 2.5 to 3 × 10^4, 2.5 to 3 × 10^5, 2.5 to 3 × 10^6, and 2.5 to 3 × 10^7 spermatozoa/mL.

In experiment 3, to investigate the effect of spermatozoa from different species of mammals on *in vitro* maturation of oocytes, *in vitro* maturation was performed in a medium containing porcine, bovine and human spermatozoa.

In experiment 4, to evaluate which stages of oocytes maturation such as, GVBD, metaphase I, anaphase-telophase I and metaphase II are affected by co-culture with spermatozoa, oocytes were exposed for various periods of maturation, 0 to 24, 0 to 48, 6 to 24, and 12 to 24 h in TCM 199 supplemented with 2.5 to 3 × 10^6 spermatozoa/mL. For a total 48 h of culture another culture was performed in TCM alone before or after co-culture.

Statistical Analysis

Statistical analysis was performed with a statistical package program using χ^2 test. Statistical significance was considered at $p<0.05$.

RESULTS

Role of Spermatozoa

Table 1. Effect of co-culture with spermatozoa on *in vitro* maturation of porcine cumulus-enclosed germinal vesicle oocytes in chemically defined medium

Addition of spermatozoa	No of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A-T	MII
Without	96	17 (17.7)[a]	30 (31.3)	29 (30.2)	8 (8.3)	12 (12.5)[a]
With	107	6 (5.6)[b]	21 (19.6)	15 (14.0)	9 (8.4)	56 (52.3)[b]

GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, A-T: anaphase I-telophase I, MII: metaphase II, [a,b]Column with different superscripts were significantly different($P<0.05$).

The effect of co-culture with spermatozoa during *in vitro* maturation of porcine COCs is shown in Table 1. When the oocytes were matured in media without spermatozoa, the proportions of oocytes remained at GV and reached at metaphase II were 17.7% and 12.5%, respectively. The percentage of metaphase II was significantly ($P<0.05$) increased in the oocytes cultured in media containing spermatozoa compared to those in media without spermatozoa (52.3% vs 12.5%). However, the proportion of oocytes remained at GV were significantly ($P<0.05$) decreased in the oocytes cultured in media containing spermatozoa than those in media without spermatozoa (17.7% vs 5.6%).

Concentrations of Spermatozoa

As shown in Table 2, when the spermatozoa concentrations of the medium were changed to 2.5 to 3 $\times$ 10^3, 2.5 to 3 $\times$ 10^4, and 2.5 to 3 $\times$ 10^5 spermatozoa/mL, the percentages of oocytes reached at metaphase II stage were 15.4, 16.5 and 23.0%, respectively. Furthermore, when the concentrations of spermatozoa were increased to 2.5 to 3 $\times$ 10^6, and 2.5 to 3 $\times$ 10^7 spermatozoa/mL, the proportion of oocytes reaching metaphase II stage were significantly increased to 46.7 and 48.9% ($P<0.05$), respectively. Additionally, a significant difference in the proportion of oocytes remained at a GV stage was found between high and low concentrations of spermatozoa ($P<0.05$).

Source of Spermatozoa

Table 3 depicts whether spermatozoa from different species of mammals affect or not *in vitro* maturation of porcine oocytes. *In vitro* maturation was performed in a medium containing porcine, bovine or human spermatozoa. No significant differences in the average values of GVBD and metaphase II were observed among the spermatozoa from different species of mammals.

Period of Spermatozoa Exposure

To evaluate which stages of maturation was stimulated by spermatozoa, co-culture with spermatozoa were performed at different period. After the first 6 or 12 h of culture in TCM 199 alone, another 18 or 12 h of culture oocytes were re-incubated with TCM 199 containing spermatozoa. Another group of oocytes was cultured in media containing spermatozoa during first 24 or 48 h of culture. For a total 48 h of culture, oocytes were transferred into TCM alone after co-culture. The results obtained are shown in Table 4. No significant differences in the average values of GV and metaphase II were

Table 2. Effect of co-culture with different concentrations of spermatozoa on *in vitro* maturation of porcine cumulus-enclosed germinal vesicle oocytes in chemically defined medium

Concentration (sperm/mL)	No of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A-T	MII
Control	90	15 (16.7)[a]	29 (32.2)	17 (18.9)	14 (15.5)	15 (16.7)[a]
2.5~3× 10^3	97	17 (17.5)[a]	28 (28.9)	18 (18.6)	13 (13.0)	15 (15.4)[a]
2.5~3× 10^4	97	11 (11.4)[a]	27 (27.8)	27 (27.8)	16 (27.8)	16 (16.5)[a]
2.5~3× 10^5	74	3 (4.0)[b]	22 (29.7)	21 (28.4)	11 (14.9)	17 (23.0)[a]
2.5~3× 10^6	75	5 (6.6)[b]	14 (18.7)	14 (18.7)	7 (9.3)	35 (46.7)[b]
2.5~3× 10^7	92	3 (3.3)[b]	15 (16.3)	19 (20.7)	10 (10.9)	45 (48.9)[b]

GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, A-T: anaphase I-telophase I, MII: metaphase II, [a,b]Column with different superscripts were significantly different($P<0.05$).

Table 3. Effect of co-culture with spermatozoa from different species on *in vitro* maturation of porcine cumulus-enclosed germinal vesicle oocyte in chemically defined medium

Source of spermatozoa	No of oocytes	Maturation stage(%)				
		GV	GVBD	MI	A-T	MII
Control	97	15 (16.7)[a]	29 (32.2)	17 (18.9)	14 (15.5)	15 (16.7)[a]
Human	90	7 (7.8)[b]	17 (18.9)	9 (10.0)	11 (12.2)	46 (51.1)[b]
Porcine	101	9 (8.9)[b]	20 (19.8)	16 (15.8)	8 (7.9)	48 (47.6)[b]
Bovine	88	5 (5.7)[b]	17 (19.4)	11 (12.5)	12 (13.6)	43 (48.8)[b]

GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, A-T: anaphase I-telophase I, MII: metaphase II, [a,b]Column with different superscripts were significantly different($P<0.05$).

Table 4. Effect of various duration of spermatozoa exposure during *in vitro* maturation of porcine cumulus-enclosed germinal vesicle oocyte in chemically defined medium

Duration of exposure	No of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A-T	MII
Control	91	16 (17.6)[a]	30 (33.0)	27 (29.6)	8 (8.8)	10 (11.0)[a]
0~24h	94	5 (5.3)[b]	24 (25.5)	9 (9.6)	11 (11.7)	45 (47.9)[b]
0~48h	91	4 (4.4)[b]	16 (17.6)	15 (16.5)	10 (11.0)	46 (50.5)[b]
6~24h	90	6 (6.7)[b]	19 (21.1)	14 (15.6)	9 (10.0)	42 (46.7)[b]
12~24h	97	5 (5.2)[b]	20 (20.6)	16 (16.5)	13 (13.4)	43 (44.3)[b]

GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, A-T: anaphase I-telophase I, MII: metaphase II, [a,b]Column with different superscripts were significantly different($P<0.05$).

observed among the different period of spermatozoa exposure.

DISCUSSION

Porcine GV oocytes have been shown to complete meiotic maturation *in vitro* under suitable culture conditions. To our knowledge, this is the first report that co-culture of porcine COCs with mammalian spermatozoa during *in vitro* maturation even before fertilization was able to improve the nuclear maturation in a hormone-free, chemically defined medium. Several groups have reported beneficial effects upon maturation of GV oocytes by using follicular cell (Staigmiller and Moor, 1984) and in media supplemented with follicular fluid (Vatzias and Hagen, 1999). When serum is added to culture medium, it acts as a source of albumin that balances osmolality and scavengers of harmful molecules (Stone et al, 1988). Serum may also act as a source of growth factor, hormone and other beneficial substances that prevent premature release of cortical granules and *in vitro* zona hardening (Down et al, 1986). Because many factors are present in the follicular fluid and FCS, it is difficult to investigate which factors affect the *in vitro* nuclear maturation of oocytes. Pig oocytes can be matured in protein-free medium supplemented with gonadotropins (Abeydeera et al, 1998). The most commonly used basic culture media are TCM 199 (Wang et al, 1994) and Tyrode's solution (Abeydeera et al, 1998). Therefore, the design of this study was to evaluate the possible effects of spermatozoa exclusively without interference from other signaling molecules on maturation *in vitro* of porcine germinal vesicle oocytes in a hormone-free, chemically defined medium, TCM 199.

This study shows that more than fifty percent of COCs co-cultured with mammalian spermatozoa could reach at M II stage (Table 1), which is similar to the proportion of metaphase II stage observed in media supplemented with combination of various hormones, growth factors, serum and/or follicular fluid (Nagai et al, 2000; Vatzias and Hagen, 1999). The results of this study suggest that mammalian spermatozoa contain a substance(s) that improves the nuclear maturation of oocytes. Because spermatozoa were not capacitated, no oocyte penetrated

by spermatozoa observed. In the physiological course of the event, the oocyte is exposed to spermatozoa after reaching metaphase II stage in most mammalian species. Until penetrating spermatozoa, the second meiosis of oocyte is arrested at the metaphase II. Penetrating spermatozoa play a major role in resumption of second meiosis (Kouba et al, 2000). It is difficult to explain why spermatozoa have a beneficial component for the oocytes maturation. The maturation promoting effect of spermatozoa is evident for cumulus-free oocytes also (Kim et al, 2003), suggesting that spermatozoa may act, at least in part, directly on the oocytes itself. Several authors have reported the evidence suggesting that cAMP controls the maintenance of meiotic arrest in mammalian oocytes and that purines, e.g., hypoxanthine, in follicular fluid increase the cAMP (Bilodeau et al, 1993; Down, 1993). Maturation-promoting factor (MPF) displays a cyclic activity that peaks at metaphase (Nurse, 1990). A decrease in MPF and mitogen-activated protein kinase (MAPK) activity coincided with metaphase II exit and pronuclear formation, respectively (Liu et al, 1998). Growth factors, such as epidermal growth factor and insulin-like growth factor 1 act on cumulus-oocyte complexes from small follicles to accelerate the meiotic cell cycle of the oocytes, which may be related to increase MPF and MAPK activities during the early stages of maturation (Sakaguchi et al, 2002). Although we did not confirm the intracellular signal responsible for the cellular events leading to the oocytes maturation from germinal vesicle through M II, the present study suggest that spermatozoa triggers a cascade of sequential stages of oocyte activation. Further experiments are necessary to clarify the intracellular signal pathway induced by spermatozoa.

Spermatozoa of foreign species also could enhance porcine oocytes maturation (Table 3). Human and bovine spermatozoa, for example, activated porcine oocytes very efficiently. This result suggests that the enhancing effect of spermatozoa for oocytes maturation is not highly species-specific. This apparent lack of species-specific enhancing effect is similar to previous reports of sperm extracts activating metaphase II stage of oocytes in homologous (Stice and Robl, 1990) and heterologous species (Rybouchkin et al, 1995).

I also observed that the proportion of oocytes reaching metaphase II stage was significantly higher in high concentrations of spermatozoa than in low concentrations of spermatozoa ($P<0.05$) (Table 2). This result indicates that the addition of spermatozoa during culture of pig oocytes enhance oocyte maturation in a dose-dependent manner *in vitro*. I cannot explain what components of spermatozoa are beneficial for maturation of oocytes *in vitro*. When oocytes were cultured in the medium alone or containing low concentration of spermatozoa, the proportion of oocytes remaining in the germinal vesicle stage was significantly increased. From this data I considered that the oocytes were more sensitive to spermatozoa for the germinal vesicle breakdown stage.

Meiotic maturation of porcine oocytes was induced by culture with media containing pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG) with or without estradiol (Vatzias and Hogen, 1999). The exposure of an COCs to hormonal supplements for only 2 h enhanced germinal vesicle breakdown and meiotic maturation (Funahashi and Day, 1993). Under the presence of PMSG or hCG alone or both hormones, nearly all of porcine oocytes showed meiotic maturation by exposure of the first 20 h period (Funahashi et al, 1994). The data in this study demonstrates that the presence of spermatozoa in maturation medium during 12 h period was sufficient for the accomplishment of germinal vesicle breakdown and meiotic maturation (Table 4).

In conclusion, the present study suggests that mammalian spermatozoa have a beneficial effect for nuclear maturation *in vitro* of oocytes even before fertilization. The enhancing effect of spermatozoa for oocytes maturation *in vitro* is highly dose-dependent and not species-specific. The exact biochemical characteristic of beneficial substance(s) from spermatozoa remains to be investigated. Further studies on the beneficial substance(s) will be both of academic value in understanding the physiological interaction between the oocytes and the beneficial substance(s) and of practical importance in improving the oocytes culture system.

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