

Effect of Co-Culture with Mammalian Spermatozoa on Maturation in vitro of Porcine Cumulus-Free Germinal Vesicle Oocytes

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Abstract

The purpose of this study was to determine if the addition of spermatozoa into the culture medium could influence the nuclear maturation of denuded porcine germinal vesicle (GV) oocytes in vitro. Cumulus-oocyte complexes were collected from follicles of 3 to 5 mm in diameter. The cumulus and corona cells were removed from oocytes. Porcine denuded oocytes were cultured in tissue culture medium containing spermatozoa. After 48 h culture, oocytes were examined for the evidence of GV breakdown, metaphase I, anaphase-telophase I, and metaphase II (M II). The proportion of oocytes reaching M II stage was significantly ($P < 0.01$) increased in the oocytes cultured in media containing spermatozoa compared to those in media without spermatozoa ($31.9 \pm 1.8\%$ vs $14.9 \pm 1.0\%$). No differences in the rates of M II were observed among the different period of spermatozoa exposure nor among the spermatozoa from different species. The proportion of oocytes reaching M II stage was significantly different between high and low concentrations of spermatozoa. The present study suggests that mammalian spermatozoa contain a substance(s) that improves nuclear maturation in vitro of GV oocytes. Enhancing effect of spermatozoa for oocytes maturation in vitro 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 (M 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[23] 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[29]. In humans, however, spontaneous maturation in vitro is achieved in only 30% to 50% of oocytes[5,9]. 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 bovine serum albumin [36]. Hormonal supplement are achieved by addition of various combination of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol. Bovine [14] and rabbit[35] oocytes matured in the absence of

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gonadotropins have, in fact, shown satisfactory developmental competence. Although it is generally accepted that cumulus cells during oocytes maturation support nuclear and cytoplasmic maturation for developmental competence after fertilization, cumulus-free oocytes can complete meiotic maturation in rat[20], sheep[26], pig [33], cattle[6] and human[17] in vitro.

Recently, cumulus-intact porcine oocytes matured in a medium supplemented with protein and estradiol and without FSH in vitro have poor ability to undergo germinal vesicle breakdown and mature to M II[21]. Previous studies[10] observed that compared with the 10% rate of spontaneous maturation, addition of sperm to culture medium led to metaphase II in 45% of human germinal vesicle oocytes. However, no published data on addition of spermatozoa during in vitro maturation of porcine germinal vesicle oocytes has been reported. Therefore, the objective of present study was to examine whether in vitro nuclear maturation of denuded porcine germinal vesicle oocytes can 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 mg/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°C. The ovaries were pooled

regardless of the stage of the donors cycle. Cumulus-oocyte complexes from follicles of 3 to 5 mm in diameter were selected on the basis of their translucent appearance, good vascularization and the compact of granulosa layer and cumulus mass. Cumulus-oocyte complexes were washed four times in maturation medium. The cumulus and corona cells were removed from oocytes by treatment with phosphate buffered saline containing 0.1% hyaluronidase from bovine testes and by repeated passage through a fine pipette. A group of 10 to 15 cumulus-free oocytes were transferred into a 100 μ l maturation medium under warm paraffin oil in a polystyrene culture dish, which has been previously kept for about 4 h in a CO₂ incubator. Oocytes were cultured at 39°C under an atmosphere of 5% CO₂ and 95% air with high humidity.

Sperm preparation

Cryopreserved and ejaculated boar and bovine semens 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 NaH₂PO₄, 2.4 g Tris, 0.1 g MgSO₄, 2.6 g NaHCO₃, 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 yolk and glycerol. Freezing protocol started at room temperature, then cooled at a rate of 1.0°C/min to -70°C and plunged into liquid nitrogen. The cryopreserved semen were thawed at 37°C for about 1 min. Cryopreserved semen washed three times by centrifugation, each time at 1,200 \times g for 1 min and re-suspended in TCM to give a concentration of 5 to 6 \times 10⁶ spermatozoa/ml.

Assessment of oocytes

Oocytes were mounted after 48 h culture, then 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 the evidence of germinal vesicle breakdown (GVBD), metaphase I, anaphase-telophase I and M II.

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

The effect of co-culture with spermatozoa during in vitro maturation in cumulus-free porcine oocytes is shown in Fig. 1. When the oocytes were matured in media without spermatozoa, the proportions of oocytes remained GV and reached M II were $19.0 \pm 2.3\%$ and $14.9 \pm 1.0\%$, respectively. The rate of M II was significantly

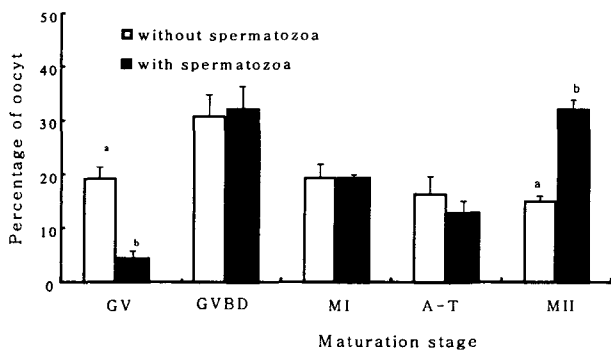


Fig. 1. Effect of co-culture with spermatozoa on in vitro maturation of porcine cumulus-free germinal vesicle oocytes in a chemically defined medium. GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, A-T: anaphase I-telophase I, MII: metaphase II. Bars with different letters are significantly different ($P < 0.01$). Values are expressed as means \pm SE. the rates of GV were significantly ($P < 0.01$) decreased in the oocytes cultured in media containing spermatozoa than those in media without spermatozoa ($4.2 \pm 1.4\%$ vs $19.0 \pm 2.3\%$).

($P < 0.01$) increased in the oocytes cultured in media containing spermatozoa compared to those in media without spermatozoa ($31.9 \pm 1.8\%$ vs $14.9 \pm 1.0\%$). However, the rates of GV were significantly ($P < 0.01$) decreased in the oocytes cultured in media containing spermatozoa than those in media without spermatozoa ($4.2 \pm 1.4\%$ vs $19.0 \pm 2.3\%$).

Maturation stage of oocytes

To evaluate which stages of maturation was affected by spermatozoa, IVM was performed in different periods of spermatozoa exposure. After the first 6 or 12 h of culture in TCM 199 alone, oocytes were re-incubated with TCM 199 containing spermatozoa for 18 or 12 h of culture. 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 1. No significant differences in the average values of GV and M II were observed among the different period of spermatozoa exposure.

Concentrations of spermatozoa

Maturation rates of oocytes in different concentrations of spermatozoa are shown in Table 2. When the spermatozoa concentrations of the medium were changed to 2.5 to 3×10^3 , 2.5 to 3×10^4 , and 2.5 to 3×10^5 spermatozoa/ml, the rates of M II stage oocytes were 15.4, 16.5 and 23.0%, respectively. Furthermore, when the concentrations of spermatozoa were increased to 2.5 to 3×10^6 , and 2.5 to 3×10^7 spermatozoa/ml, the proportion of oocytes reaching M II stage were significantly increased to 43.1 and 38.0% ($P < 0.05$), respectively. Additionally, a significant difference in oocytes remained at a GV stage found between high and low concentrations of spermatozoa ($P < 0.05$).

Source of spermatozoa

Fig. 2 depicts whether spermatozoa from different

Table 1. Effect of spermatozoa exposure during various periods on in vitro maturation of porcine cumulus-free germinal vesicle oocytes in a chemically defined medium

Period of spermatozoa exposure	Total no. of oocytes examined	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
Control	96	14 (14.6) ^a	31 (32.3)	18 (18.7)	16 (16.7)	17 (17.7) ^a
0-24 h	100	5 (5.0) ^b	32 (32.0)	17 (17.0)	13 (13.0)	33 (33.0) ^b
0-48 h	94	4 (4.3) ^b	30 (31.9)	18 (19.1)	12 (12.8)	30 (31.9) ^b
6-24 h	91	6 (6.6) ^b	27 (29.6)	14 (15.4)	12 (13.2)	32 (35.2) ^b
12-24 h	72	2 (2.8) ^b	8 (11.1)	14 (19.4)	17 (23.6)	31 (43.1) ^b

GV: germinal vesicle, GVBD: germinal vesicle break down, MII: metaphase I, A-T: anaphase I-terophase I, MIII: metaphase II.
^{a,b}Column with different superscripts were significantly different (P <0.05).

Table 2. Effect of spermatozoa concentrations on in vitro maturation of porcine cumulus-free germinal vesicle oocytes in a chemically defined medium

Concentration of spermatozoa	Total no. of oocytes examined	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
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)	19 (19.6)	15 (15.4) ^a
2.5-3 × 10 ⁴	97	11 (11.4) ^{ab}	27 (27.8)	27 (27.8)	16 (27.8)	16 (16.5) ^a
2.5-3 × 10 ⁵	74	3 (4.0) ^{bc}	22 (29.7)	21 (28.4)	11 (14.9)	17 (23.0) ^a
2.5-3 × 10 ⁶	72	2 (2.8) ^c	8 (11.1)	14 (19.4)	17 (23.6)	31 (43.1) ^b
2.5-3 × 10 ⁷	92	3 (3.3) ^c	10 (10.9)	18 (19.5)	26 (28.3)	35 (38.0) ^b

GV: germinal vesicle, GVBD: germinal vesicle break down, MII: metaphase I, A-T: anaphase I-terophase I, MIII: metaphase II.
^{a,b,c}Column with different superscripts were significantly different(P <0.05).

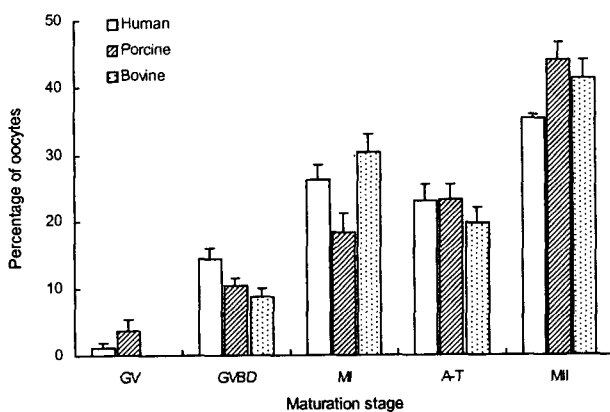


Fig. 2. Effect of spermatozoa from different species on in vitro maturation of porcine cumulus-free germinal vesicle oocytes in a chemically defined medium.

GV: germinal vesicle, GVBD: germinal vesicle break down, MII: metaphase I, A-T: anaphase I-terophase I, MIII: metaphase II. Values are expressed as means±SE.

species of mammals affect 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 M II were observed among the spermatozoa from different species of mammals.

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 with spermatozoa during in vitro maturation was able to improve the nuclear maturation of porcine oocytes in a hormone-free, chemically defined medium. We have previously observed that the maturation rate of

human GV oocytes from stimulated cycles was not affected by presence or absence of cumulus except that the maturation was more synchronous in oocytes with cumulus than in those without cumulus[17]. A novel method of growth factor and certain protein storage and release by an apparent apocrine-like mechanism was demonstrated in human follicular and cumulus cells[3]. Some of these proteins are expressed and selectively distributed in mature oocytes and developing embryo, which is suggestive of their role during embryogenesis [2]. Several groups have reported beneficial effects upon maturation of porcine oocytes by using follicular fluid [30] and in media supplemented with FCS[36], and hormone[34]. When serum is added to culture medium, it acts as a source of albumin that balances osmolality and scavengers of harmful molecules[15]. 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[8]. Because many factors are present in the cumulus cell mass, 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[1]. The most commonly used basic culture media are TCM 199[31] and Tyrode's solution[17]. 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 GV oocytes in a chemically defined medium, TCM 199.

We found that the rate of M II was significantly ($P < 0.01$) increased in the oocytes cultured in media containing spermatozoa compared to those in media without spermatozoa, whereas the rate of GV was significantly ($P < 0.01$) decreased in the oocytes cultured in media containing spermatozoa even before fertilization than those in media without spermatozoa (Fig. 1). The results of this study suggest that mammalian spermatozoa contain a substance(s) that improves the rate of nuclear mat-

uration. In the physiological course of the event, the oocyte is exposed to spermatozoa after reaching M II stage in most mammalian species. Until penetrating spermatozoa, the second meiosis of oocyte is arrested at the M II. Penetrating spermatozoa play a major role in resumption of second meiosis[28]. 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, suggesting that spermatozoa may act, at least in part, directly on the oocytes itself. The zona pellucida allows the passage of molecules as large as 150 kDa in the mouse[18], because they still possess cumulus cell projection embedded in the zona pellucida[16], from which both inhibitory or stimulatory signals may be transferred to the oocytes. The porcine zona may be somewhat similar in this regard. We consider that relatively small and instructional molecules pass from spermatozoa to the oocytes, where they inhibit meiosis. It has been reported that different triggers induced the sequential stages of oocytes maturation from germinal vesicle through M II toward 2 pronuclei, after fertilization. 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[4,7]. The increase in maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities may be necessary for the onset of germinal vesicle breakdown and metaphase progression during oocyte maturation and meiotic arrest[11,32]. MPF displays a cyclic activity that peaks at metaphase[22]. A decrease in MPF and MAPK activity coincided with M II exit and pronuclear formation, respectively[19,25]. Although we did not confirm the intracellular signal responsible for the cellular events leading to the oocytes maturation from germinal vesicle to MII, present study suggest that spermatozoa trigger a cascade of sequential stages of oocyte activation. Further study is necessary to clarify the intra-

cellular signal pathway induced by spermatozoa.

Spermatozoa of foreign species could also enhance porcine oocytes maturation (Fig. 2). 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 M II stage of oocytes in homologous [27] and heterologous species[24].

We also observed that the proportion of oocytes reaching M 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. We 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 we 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[34]. The exposure of an oocytes-cumulus complex to hormonal supplements for only 2 h enhanced germinal vesicle breakdown and meiotic maturation[7]. 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[12,13]. 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 1).

In conclusion, the present study suggests that mam-

malian 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.

Acknowledgement

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초록 : 난구세포가 제거된 돼지 미성숙 난자의 체외성숙에 포유동물 정자가 미치는 영향

김병기 · 강성룡 · 임자옥 · 한창희 · 이복규 · 이광선
(동의대학교 자연과학대학 분자생물학전공)

본 연구는 난구세포가 제거된 돼지 미성숙 난자와 포유동물 정자의 공배양이 체외핵성숙에 미치는 영향을 조사하기 위하여 실시하였다. 난구세포가 부착된 미성숙난자를 직경 3-5 mm 난포로부터 채취하여 난구세포를 제거한 후 정자가 첨가된 tissue culture medium 199에서 배양하였다. 배양 후 48시간에 난자의 핵성숙을 핵막붕괴, 제일차 감수분열 중기, 제일차 감수분열 후기-말기, 제이차감수분열 중기로 판정하였다. 배양 후 제이차감수분열 중기로 성숙한 난자의 비율이 무첨가구에 비하여 정자가 첨가된 배양액에서 성숙시킨 미성숙난자에서 유의적인($P < 0.01$) 증가가 있었다($31.9 \pm 1.8\%$ vs $14.9 \pm 1.0\%$). 난자의 성숙 단계에 따른 정자의 노출 시간과 정자가 유래한 종에 따라서는 차이가 없었다. 그러나 정자의 첨가농도에 따라 유의적인 차이가 인정되었다. 본 연구는 포유동물의 정자는 미성숙난자의 체외성숙을 촉진하는 물질을 갖고 있으며, 촉진 효과는 농도에 의존하는 것으로 암시하고 있다.