Effect of Co-culture with Spermatozoa on the Resumption of Meiosis in Porcine Germinal Vesicle Oocytes Arrested with Meiotic Inhibitors

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ABSTRACT

***In vitro* maturation of porcine immature cumulus-enclosed oocytes can be enhanced by co-incubation with spermatozoa even before fertilization. The aim of this study was to determine whether the addition of spermatozoa into the culture medium can stimulate the meiosis resumption of porcine cumulus-enclosed oocytes arrested at germinal vesicle (GV). Cumulus-enclosed oocytes (CEOs) were collected from follicles of 3 to 5 mm diameter. Porcine CEOs were cultured in tissue culture medium containing various meiosis inhibitors and spermatozoa. Oocytes were examined for evidence of GV and GV breakdown after 24 h culture. After 24 h culture 43.8% of oocytes cultured in only TCM 199 remained at GV stage whereas 56.2% of oocytes were able to resume meiosis. When porcine CEOs were cultured in the medium with meiosis inhibitor such as, dibutyryl cAMP (dbcAMP) and forskolin (Fo), more than 90 % of oocytes were not able to resume meiosis. However, co-culture of porcine CEOs with spermatozoa was able to overcome the inhibitory effect of dbcAMP and Fo. Irrespective of the presence of 3-isobutyl-1-methylxanthine (IBMX), no difference was observed in the proportion of oocyte reached germinal vesicle breakdown (GVBD). The present study suggests that dbcAMP and Fo prevent the spontaneous maturation of competent oocyte in culture after isolation from follicles and that mammalian spermatozoa contain a substance(s) that improves meiosis resumption *in vitro* of porcine cumulus-enclosed oocytes.**

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

INTRODUCTION

The maturation of mammalian oocytes includes important nuclear changes, which is considered as the re-initiation and completion of the first meiotic division from prophase I to metaphase II. Several factors, such as high levels of cAMP and hypoxanthine (Hx) in the compartment surrounding the oocyte, prevent largely meiotically competent oocytes from resuming meiosis spontaneously (Downs *et al.*, 1985; Eppig *et al.*, 1985; Törnell and Hillensjö, 1993). In addition, meiosis-inhibiting substances from cumulus cells contribute to meiotic arrest at the GV stage (Andersen *et al.*, 1999). *In vivo*, LH triggers the resumption of meiosis and thus induces the dormant nucleus to resume meiosis, cytologically visible by germinal vesicle breakdown (GVB). Because oocyte has no LH receptors (Dekel, 1988) or the direct association of cumulus cells with the oocyte (Eppig *et al.*, 1997), the activating action of LH on the oocyte appears to be indirect. When the cumulus cells were removed from oocytes in large follicles, resumption of meiosis initiate rapidly because the intracellular concentrations of cAMP in oocytes decline (Downs, 1995[a]). Keeping concentrations of cAMP high in the oocyte, either by inhibition of phosphodiesterase activity or by culture of oocytes in the presence of membrane-permeable cyclic nucleotides, inhibits resumption of meiosis of naked oocytes (Törnell and Hillensjö, 1993; Downs, 1995[b]). With regard to activation of meiotic maturation, it has been well known that porcine oocytes cultured in a medium without gonadotropin *in vitro* have poor ability to undergo GVBD and mature metaphase II (Nagai *et al.*, 2000). However, Kim *et al.*, (2003) and Kim (2004) showed that co-culture of immature porcine oocyte with spermatozoa was dose-dependently able to enhance the oocyte maturation *in vitro* in denuded or cumulus enclosed oocytes, even before oocytes were fertilized. Previous studies (Farhi *et al.*, 1997) also observed that compared with the 10% rate of spontaneous maturation, addition of sperm to culture medium led to metaphase II in 45% of human GV oocytes. It is therefore of interest to investigate whether the action of agents such as, dbcAMP, IMBX, Hx and

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Fo, mediating meiotic arrest by inducing adenylate cyclase and by increasing membrane-permeable cyclic nucleotides (Urner, 1983), can be also overcome by addition of spermatozoa *in vitro*. Since oocytes rapidly resume maturation when cAMP levels drop, it is important to examine whether co-culture with spermatozoa influences the kinetics of meiotic maturation in porcine oocytes overcoming the meiotic block. It is also still completely unknown whether co-culture of oocytes with spermatozoa affects nuclear maturation events in mammalian oocytes. Therefore, the present study was performed to investigate the effect of spermatozoa on the meiosis resumption of porcine cumulus enclosed oocytes arrested at GV stage.

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 μg/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. 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. CEOs were washed four times in maturation medium. A group of 10 to 15 CEOs were transferred into a 100 μL maturation medium under warm paraffin oil in a polystyrene culture dish, which had been previously kept for about 4 h in a CO_2 incuba- tor. Oocytes were cultured at 39℃ under an atmosphere of 5% CO_2 and 95% air with high humidity.

Sperm Preparation

Porcine epididymis from random breeds were collected immediately post mortem at a local slaughterhouse and transported to the laboratory within 2 h in ice. The surface of epididymis was washed three times with physiological saline solution. The epididymal fluid was aspirated from cauda of epididymis and centrifuged at 1,500 g for 10 min. The supernatant was removed. The pelleted spermatozoa were gently suspended in TCM and washed three times by centrifugation. After washing, the final sperm pellet was resuspended in the same medium to give a sperm concentration of $2\sim3\times10^6$ spermatozoa/mL.

Assessment of Oocytes

Oocytes were mounted after 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.

Statistical Analysis

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

RESULTS

The distribution of nuclear morphology of porcine cumulus-enclosed oocytes at 24 h when cultured in the presence of hypoxanthine (Hx) and co-cultured with spermatozoa is shown in Fig. 1. After 24 h culture 43.8% of oocytes (71/162) cultured in only TCM 199 remained at GV stage, whereas 56.2% of oocytes were able to resume meiosis. When oocytes were cultured in medium supplemented with 1.5 mM or 3.0 mM Hx, only 28.8% and 25.2% of oocytes were able to reach at GVBD stage, respectively. Although meiosis resumption of porcine cumulus-enclosed oocytes was inhibited by Hx, 85.4% of oocytes were able to resume meiosis by co-culture of oocytes with spermatozoa.

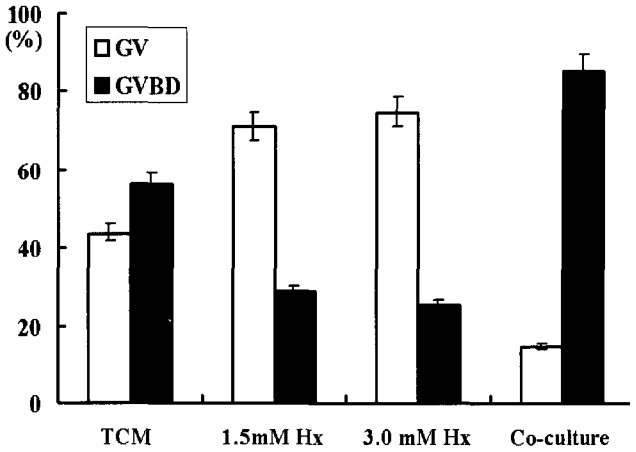

Fig. 1. The effect of co-culture with spermatozoa on meiosis resumption of porcine germinal vesicle oocytes arrested with hypoxanthine (Hx.). Values are expressed as the mean ± SEM. Bars with different letter in the germinal vesicle breakdown are significantly different ($p<0.05$).

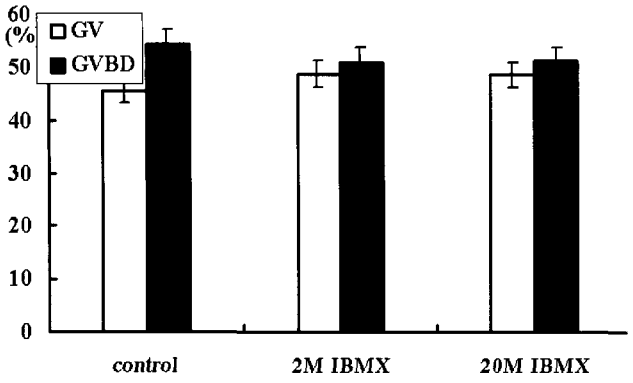

Fig. 2. The effect of 3-isobutyl-1-methylxanthine (IBMX) on meiosis resumption of porcine germinal vesicle oocytes. Value are expressed as the mean ± SEM.

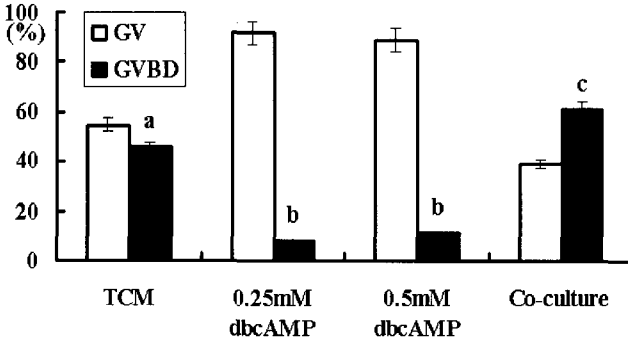

Fig. 3. The effect of co-culture with spermatozoa on meiosis resumption of porcine germinal vesicle oocytes arrested with dibutyryl cAMP (dbcAMP). Value are expressed as the mean ± SEM. Bars with different letter in the germinal vesicle breakdown are significantly different ($p<0.05$).

As shown in Fig. 2, after 24h culture of porcine CEOs in TCM 199, 45.6% of oocytes remained at GV stage, whereas 54.4% of oocytes were able to resume meiosis. When porcine cumulus-enclosed immature oocytes were cultured in the medium containing 2 M or 20 M IBMX, germinal vesicle breakdown was observed in 51.2% and 51.3% of oocytes, respectively. No difference between absence and presence of IBMX was observed in the proportion of oocyte reached at GVBD stage.

Fig. 3 depicts that after 24h culture, most of the oocytes cultured in the presence of 0.25 mM and 0.5 mM dbcAMP (91.9% and 89.1%, respectively) were arrested at the GV stage, higher than what is observed in TCM alone. When oocytes were co-cultured with spermatozoa even before fertilization, it was observed that 61.0 % of oocytes were able to resume meiosis in spite of an inhibitory effect of dbcAMP.

As shown in Fig. 4, the presence of forskolin during *in vitro* maturation culture prevented porcine cumulus enclosed-oocytes from resuming meiosis. However, co-culture of porcine CEOs with spermatozoa was able to overcome the inhibitory effect of forskolin.

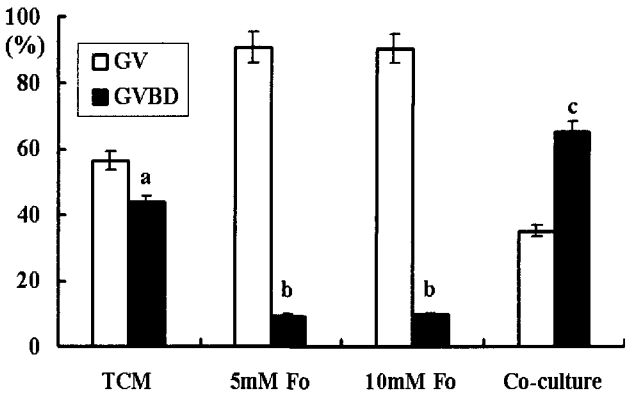

Fig. 4. The effect of co-culture with spermatozoa on meiosis resumption of porcine germinal vesicle oocytes arrested with forskolin (Fo). Value are expressed as the mean ± SEM. Bars with different letter in the germinal vesicle breakdown are significantly different ($p<0.05$).

DISCUSSION

Several groups have reported beneficial effects upon porcine oocytes nuclear maturation by using epidermal growth factor (EGF) (Ding and Foxcroft, 1994), cysteamine (Yamauchi and Nagai, 1999), follicular fluid (Hegele-Hartung *et al.*, 2001) and hormones (Zuelke and Brackett, 1990). The design of this chemically defined medium was justified by my objective to observe the possible effects of spermatozoa exclusively without interference from other signaling molecules. This study examined the effect of co-culture with speramtozoa on meiotic maturation of porcine oocytes, arrested with either dbcAMP, Hx or Fo in prophase of meiosis I. Current study are showing that co-culture with speramtozoa was capable of inducing resumption of meiotic maturation in the presence of various meiotic inhibitors. Intact spermatozoa possessed oocyte meiosis-enhancing ability, whereas triton treated-spermatozoa did not (unpublished data). The most notable difference between these two types of spermatozoa was whether plasma membrane is present or not. This result suggests that spermatozoa membrane contain a component(s) that enhances the nuclear maturation *in vitro* of porcine GV oocytes.

It is difficult to explain what component(s) of spermatozoa membrane contributed to enhancing nuclear maturation *in vitro*. In mammals two meiosis activating sterols (MAS) have been found to activate meiotic resumption in mouse oocytes *in vitro*. Follicular fluid (FF)-MAS was extracted from human preovulatory follicular fluid and testicular (T)-MAS from bull testicular tissue. Adult mammalian testes mainly contain T-MAS, whereas T-MAS and FF-MAS are often found in equal concentration in ovaries (Grøndahl *et al*, 1998). In mouse

oocytes arrested with hypoxanthine, both T-MAS and FF-MAS overcome the inhibitory effect of hypoxanthine and induce resumption of meiosis in dose-dependent way (Guoliang *et al.*, 1994). Recently it was discovered that human semen contains T-MAS and the major source resulted from the spermatozoa. Previous study found that enhancing effect of spermatozoa was a highly dose-dependent (Kim *et al.*, 2003 Kim, 2004). One possible explanation is that T-MAS of spermatozoa may play the important role in resuming meiosis of oocyte.

On one hand, when protein, glycoproteins and sialoproteins on chimpanzee spermatozoa surface membrane were analyzed by 1D or 2D SDS-PAGE system, the overall common proteins were composed of at least fifty bands (Young *et al.*, 1985). In contrast, when plasma-lemmal proteins that were extracted with detergent from human spermatozoa membrane were analyzed by isoelectric focusing-PAGE, 64 proteins were visualized by silver staining (Xu *et al.*, 1994) As sperm transit through the genital tracts and interact with the luminal fluid, specific domains of their plasma membrane are remodeled by the binding of epididymal secretary proteins and by enzymatic processing (Dacheux *et al.*, 1989; Nancy *et al.*, 2000). Stable surface compounds on the spermatozoa throughout epididymal transit have also been identified in chimpanzees (Dacheux *et al.*, 1989) and swine (Nancy *et al.*, 2000). Boar spermatozoa have at least 14 distinct sperm-specific membrane proteins, which localize to the cell surface overlying all major regions of the spermatozoa (Nancy *et al.*, 2000). Determining what components of spermatozoa membrane play a role in oocytes meiosis is not possible without further study.

In rodent (Cho *et al.*, 1974; Downs, 1993) and pig (Mattioli *et al.*, 1994) the high concentration of cAMP can block the spontaneous meiotic maturation of oocytes *in vitro*. The total levels of cAMP found in the oocyte depend primarily on the rate of synthesis by adenylate cyclase and the rate of degradation by phosphodiesterases. The concentration of intracellular cAMP can be elevated by addition of membrane-permeable analogue of cAMP such as dbcAMP (Mattioli *et al.*, 1994), or by increasing the level of cAMP by activators of the adenylate cyclase such as forskolin (Downs, 1993). The presence of 1mM dbcAMP for 20 h completely inhibited the meiotic resumption of porcine cumulus-enclosed ococytes (Schultz *et al.*, 1983). In agreement with previous studies (Schultz *et al.*, 1983), present study showed that presence of dbcAMP or forskolin during 24h culture prevented resuming meiosis of porcine CEOs. Present study observed that co-culture of porcine GV oocytes with spermatozoa could also induced GVBD of porcine CEOs maintained in meiotic arrest with dbcAMP or forskolin.

While the cAMP content of intact follicle and oocyte-cumulus cell complexes rises after stimulation by gonadotropin, the cAMP level in the oocytes decrease, even though the gap junctional communication between the oocyte and cumulus cells is apparently not reduced (Hubbard, 1986). A decrease in cAMP content was not detected before either spontaneous or gonadotrophin-induced maturation of hamster oocytes (Downs *et al.*, 1988). Therefore, the maturation-inducing signal generated by cumulus cells appears to be able to bypass the meiosis-arresting action of cAMP (Eppig and Downs, 1987). Although the data of this study can not clearly explain the signal pathway of spermatozoa for resumption of meiosis, one explanation for this observation is that oocytes become stimulated to secrete or hydrolyze their cAMP by a signal produced by the cumulus cells in response to spermatozoa and/or that a positive factor released from the spermatozoa acts on the oocyte to trigger the resumption of meiosis despite the continued presence of an inhibitor. Further studies are needed to understand the physiological and cellular mechanism involved in nuclear maturation of mammalian oocyte by spermatozoa. Gonadotrophin or EGF induces maturation of mouse oocytes maintained in meiotic arrest with dbcAMP or hypoxanthine (Downs *et al.*, 1988; Eppig and Downs, 1987). Preventing the degradation of cAMP by means of treatment with phosphodiesterases inhibitors such as IBMX can also transiently delay meiotic resumption (Sirard and First, 1988). A transient effect of IBMX on meiotic arrest was observed after incubating bovine CEOs with IBMX for 8 h; however, this effect was not noticeable after 24 h In addition, IBMX is more effective in preventing the resumption of meiosis in zona-free oocytes than in CEOs (Bilodeau *et al.*, 1993).

In conclusion, the present study provides the evidences that porcine spermatozoa contain a substance(s) that enhances the nuclear maturation even before fertilization.

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