Effect of Epididymal Fluid Fractionated by Chromatography on *In Vitro* Maturation of Porcine Follicular Oocytes

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ABSTRACT

The aim of this study was to investigate what protein(s) of porcine epididymal fluid (pEF) are able to enhance the nuclear maturation of porcine germinal vesicle (GV) oocytes *in vitro*. Proteins of pEF were fractionated by affinity, ion exchange, and gel filtration chromatography. Porcine cumulus-oocytes complexes (COC) from follicles were cultured in tissue culture medium (TCM 199) containing various fractions obtained by chromatography. Porcine COCs were also cultured in TCM 199 containing various meiosis inhibitors and pEF. After 24 or 48 h culture, oocytes were examined for evidence of GV breakdown, metaphase I, anaphase-telophase I, and metaphase II. When porcine COCs were cultured in the medium with meiosis inhibitor such as, dibutyryl cAMP (dbcAMP) and forskolin (Fo), more than 80% of oocytes were unable to resume meiosis. However, porcine COCs supplemented with pEF were able to overcome the inhibitory effect of dbcAMP and Fo. Maturation rate of oocytes was significantly ($p<0.05$) increased in the media supplemented with cationic protein(s) during *in vitro* maturation than in those with anionic protein(s) (44.1% vs 20.0%). When oocytes were cultured in the TCM 199 with fractions obtained by gel filtration, the maturation rate of oocytes was significantly ($p<0.05$) higher in fraction 11 containing 18 kDa than other fractions. The present study suggests that 1) dbcAMP and Fo prevent the spontaneous maturation of oocyte after isolation from follicles, and that pEF contain a substance(s) that improves meiosis resumption *in vitro* of porcine COCs, 2) cationic 18 kDa protein(s) are responsible for promotion of MII stage.

(Key words : Chromatography, *In Vitro* maturation, Meiosis inhibitor, Porcine epididymal fluid)

INTRODUCTION

In most mammals, the oocytes are arrested at the diplotene stage of the first meiotic cycle before ovulation. *In vivo*, resumption of meiotic division in oocytes normally occurs just prior to ovulation. However, Pincus *et al.* (1935) observed the spontaneous resumption of meiosis in mammalian oocytes released from follicular environment and cultured under suitable condition. In rodents, spontaneous oocyte maturation is achieved in >95% of oocytes removed from their follicular environment (Vanderhyden *et al.*, 1990). In humans, however, spontaneous maturation *in vitro* is achieved in only 30% to 50% of oocytes (Edwards, 1965; Zheng and Sirard, 1992). Protein and hormonal supplements are two principal factors known to influence the maturation process *in vitro* within various mammalian species. In fact, maturation medium are generally supplemented with protein, such as FBS and BSA.

Gonadotropins added to maturation media enhance oocyte maturation as shown by improved completion of nuclear maturation, fertilizability, and developmental ability (Kaplan *et al.*, 1978; Wang and Niwa, 1995). Porcine oocytes matured in a medium supplemented with protein and estradiol but without FSH have poor ability to undergo germinal vesicle breakdown and to enter the metaphase II (Nagai *et al.*, 2001).

It has been reported that GV oocytes of human (Farhi *et al.*, 1997) and porcine cumulus-free (Kim *et al.*, 2003) or cumulus-enclosed germinal vesicle oocytes (Kim *et al.*, 2008) could be enhanced by co-incubation of spermatozoa even before fertilization during *in vitro* maturation. Previous studies were showing that co-culture with speramtozoa was capable of inducing resumption of meiotic maturation in the presence of various meiotic inhibitors such as, dbcAMP and Fo (Kim, 2005) and that the membrane of spermatozoa from adult epididymis have a substance(s) that can enhance IVM of oocytes (Kim, 2004). As sperm transit through the epididymis and interact with the luminal fluid, specific domains of their plasma membrane are remodeled by the binding of epididymal secretory proteins and by enzymatic processing (Antczak *et al.*, 1997). It is likely that changes in the composition of the sperm membrane are induced by exposure to the specific intraluminal environ-

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ment. Important components in this intraluminal environment are proteins secreted by the epididymal epithelium. These proteins may bind to the sperm surface and/or modify the structure or the arrangement of the existing membrane molecules (Fouchecourt *et al.*, 1999). Previous study revealed the evidence that the supplementation of pEF during maturation of oocyte enhanced porcine oocytes nuclear maturation in a dose-dependent manner *in vitro* (Kim, 2009; Yim *et al.*, 2006). However, the proportion of oocytes reaching at metaphase II was significantly decreased in the oocytes cultured in media containing trypsin-treated pEF compared with intact pEF (Yim *et al.*, 2008). This result suggests that protein(s) of pEF may be capable to promote nuclear maturation *in vitro* of germinal vesicle oocytes.

Based on the previous findings, this study was to investigate the role of the pEF protein fractions obtained by chromatography on *in vitro* maturation of porcine GV oocytes under chemically defined culture condition.

MATERIALS AND METHODS

Culture Medium

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

The basic medium used in this experiment was TCM 199 supplemented with 100 IU/ml penicillin-G, and 100 μg/ml streptomycin sulfate (pH 7.3). The medium used in this study was modified by supplementation of protein fractions obtained from pEF.

Preparation of Epididymal Fluid

The epididymis were obtained at a local slaughter house and transported to the laboratory at 0~5℃ in 0.85% saline solution supplemented with 100 IU/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. Epididymal fluid was aspirated from cauda of epididymis under aseptic procedures. Spermatozoa were separated from the aspirated fluid by centrifugation at 15,000 ×g for 15 min and supernatant of epididymal fluid was collected and used directly or stored at −80℃ with same volume of glycerol for future use.

Chromatography of Porcine Epididymal Fluid

Affinity Chromatography

The pEF was dialyzed extensively against 20 mM Tris-HCl buffer (pH 7.3) containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ (Con A binding buffer). The dialyzed samples were passed through con A Sepharose 4B column (Amersham Biosciences). The unbound proteins were washed with Con A binding buffer and bound proteins were washed with gradient of Con A elution buffer (Con A binding buffer with 0.5 M α-D-methylglucoside). Thus bound proteins were eluted and unbound protein were used the next step chromatography.

Anion-Exchange Chromatography

The unbound protein samples from affinity chromatography were dialyzed extensively against 20 Mm Tris-HCl buffer (pH 7.4). Then the dialyzed samples were passed through a column of DEAE Sepharose Fast Flow (Pharmacia Biotech) and unbound proteins were washed with 20 mM Tris-HCl buffer (pH 7.4) using gradients of 1 M NaCl. Each fractions containing protein were dialyzed against 25 mM HEPES buffer (pH 7.4).

Cation-Exchange Chromatography

The unbound protein samples from affinity chromatography were dialyzed extensively against 50 mM phosphate buffer (pH 7.0). Then the dialyzed samples were passed through a column of CM Sepharose Fast Flow (Pharmacia Biotech) and unbound proteins were washed with 50 mM phosphate buffer (pH 7.0) using gradients of 1 M NaCl. Each fractions containing protein were dialyzed against 25 mM HEPES buffer (pH 7.4).

Gel Filtration Chromatography

The pEF protein(s) were dialyzed against 25 HEPES buffer solution (pH 7.4). Then the dialyzed samples were passed through a column of Superdex 200 prep. Grade (Amersham Biosciences) and the proteins were washed with 25 mM HEPES buffer (pH 7.4). Protein concentration of each fraction was determined by the method of Lowry.

Oocytes Preparation and *In Vitro* Maturation

Porcine ovaries were collected from pubertal gilts at a local slaughterhouse and carried to the laboratory at 30~35℃ in 0.85% saline solution supplemented with 100 IU/ml potassium penicillin-G and 100 μg/ml streptomycin sulfate. The oocytes were aspirated from follicles with a diameter of 3~6 mm and pooled in 10 ml test tubes and kept stable in a water bath at 37℃. The oocytes were collected and washed three times with the maturation medium TCM 199 supplemented with 100 IU/ml penicillin G, and 100 μg /ml streptomycin sulfate (pH 7.3) under mineral oil(m 8401; Sigma) in petri dish and cultured in a CO_2 incubator (5% CO_2 in air at 39℃) for 24 or 48 hr.

Assessment of Nuclear Maturation

At the end of the culture all cumulus cells were removed by fine pipette. The oocytes, mounted on slides and covered by covers lips supported by paraffin wax posts, were fixed in acetic-alcohol (1 : 3) for 48 to 72 hr and then stained with aceto-orcein. Nuclear stage was

assorted as germinal vesicle, germinal vesicle breakdown, Metaphase I (MI), and Metaphase II (MII). Degenerated oocytes were not included in the analysis.

Statistical Analysis

Statistical analysis was performed with a standard computerized statistics program using χ^2 test. A probability of $p<0.05$ was considered statically significant.

RESULTS

Intact Porcine Epididymal Fluid Overcomes Inhibitor-Induced Meiotic Arrests of Porcine Cumulus-Oocyte Complexes

In agreement with previous studies, current study has observed that the presence of dbcAMP or forskolin during 24 h culture prevented porcine COCs from resuming meiosis. The distribution of nuclear morphology of oocytes at 24 h when cultured in the absence (control medium) or presence of 1 mM dbcAMP (referred as AMP-medium) or 5 mM forskolin (referred as Forsk-medium) is shown in Table 1. After 24 h culture, most of the oocytes cultured in the presence of dbcAMP (88.8%) and forskolin (81.0%) were arrested at the GV stage, which is higher than TCM 199 alone (control medium). When oocytes were cultured in the AMP-medium or Forsk-medium supplemented with pEF, it was observed that 60.0 and 62.1% of oocytes were able to resume meiosis, respectively. Thus, intact pEF appeared to overcome the meiotic arrest caused by the elevated cAMP in the oocytes, resulting in the enhanced maturation of oocytes with GVBD.

Table 1. Effect of porcine epididymal fluid on meiosis resumption *in vitro* of porcine germinal vesicle oocytes arrested by forskolin or dbcAMP in chemically defined medium

Treatment	No. of oocytes	Maturation stage (%)	
		GV	GVBD
Control	92	58 (63.0)	34 (37.0)[a]
Intact pEF	107	38 (35.5)	69 (64.5)[b]
Forskolin	100	81 (81.0)	19 (19.0)[c]
dbcAMP	107	95 (88.8)	12 (11.2)[c]
Forskolin + pEF	103	39 (37.9)	64 (62.1)[b]
dbcAMP + pEF	110	44 (40.0)	66 (60.0)[b]

pEF : porcine epididymal fluid, GV: germinal vesicle, GVBD: germinal vesicle breakdown.
Column with different letters (a, b, c) were significantly different ($p<0.05$).

Effect of Porcine Epididymal Fluid Fractions Obtained by Anion-Exchange Chromatography

In order to find whether anionic proteins or cationic proteins in pEF can enhance the maturation *in vitro* of porcine immature oocytes, the pEF proteins obtained by affinity chromatography were separated into anionic protein(s) (DEAE sepharose bound) and cationic protein(s) (DEAE sepharose unbound). As shown Table 2, out of 197 porcine immature oocytes matured in TCM 199 with intact pEF, 45.7% of the oocytes reached at M II stage. When porcine oocytes cultured in the medium with unbound protein(s) (cationic protein), similar result was found. However, when porcine oocytes cultured in the medium with bound protein(s) fraction 1 and 2 (anionic protein), only 20.0% and 21.4% of oocytes were able to reach at MII stage, respectively.

Effect of Porcine Epididymal Fluid Fractions Obtained by Cation-Exchange Chromatography

In order to confirm the cationic protein in pEF can enhance the maturation *in vitro* of porcine immature oocytes, the pEF proteins obtained by affinity chromatography were separated into anionic protein(s) (CM sepharose unbound) and cationic protein(s) (CM sepharose bound). As shown Table 3, out of 84 porcine immature oocytes matured in TCM 199 with intact pEF, 47.7% of the oocytes reached at MII stage. When

Table 2. Effect of porcine epididymal fluid fractions obtained by anion-exchange chromatography on *in vitro* maturation of porcine germinal vesicle oocytes

Fractions	No. of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A~T	MII
Control	84	12 (14.3)	44 (52.3)	8 (9.6)	4 (4.8)	16 (19.0)[a]
Intact pEF	197	16 (8.1)	68 (34.5)	14 (7.1)	9 (4.6)	90 (45.7)[b]
Unbound	68	5 (7.4)	26 (38.2)	5 (7.4)	2 (2.9)	30 (44.1)[b]
Bound Fr #1	70	10 (14.3)	41 (58.6)	4 (5.7)	1 (1.4)	14 (20.0)[a]
Bound Fr #2	70	9 (12.9)	40 (67.1)	5 (7.2)	1 (1.4)	15 (21.4)[a]

pEF : porcine epididymal fluid, GV: germinal vesicle, GVBD: germinal vesicle breakdown.
MI : metaphase I, A~T : anaphase-telophase, MII : metaphase II, Fr : fraction.
Column with different letters (a, b) were significantly different ($p<0.05$).

Table 3. Effect of porcine epididymal fluid fractions obtained by cation-exchange chromatography on *in vitro* maturation of porcine germinal vesicle oocytes

Fractions	No. of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A~T	MII
Control	70	15 (21.4)	30 (43.0)	5 (7.1)	5 (7.1)	15 (21.4)[a]
Intact pEF	84	4 (4.8)	27 (32.1)	7 (8.3)	6 (7.1)	40 (47.7)[b]
Unbound	115	16 (13.8)	54 (47.0)	11 (9.6)	7 (6.1)	27 (23.5)[a]
Bound Fr #1	110	13 (11.9)	57 (51.8)	8 (7.2)	7 (6.4)	25 (22.7)[a]
Bound Fr #2	118	8 (6.9)	43 (36.4)	9 (7.6)	3 (2.5)	55 (46.6)[b]

pEF : porcine epididymal fluid, GV: germinal vesicle, GVBD: germinal vesicle breakdown.
MI : metaphase I, A~T : anaphase-telophase, MII : metaphase II, Fr : fraction.
Column with different letters (a, b) were significantly different ($p<0.05$).

porcine oocytes cultured in the medium with unbound protein(s) (mostly anionic protein), result was similar to control group (TCM 199 alone). However, when porcine oocytes cultured in the medium with bound protein(s) fraction 2 (cationic protein), the proportion of oocytes reached at MII stage was significantly ($p<0.05$) increased than those of oocytes cultured in control medium.

Effect of Porcine Epididymal Fluid Fractions Obtained by Gel Filtration Chromatography

The proteins in pEF were separated by gel filtration chromatography using superdex 200 prep. According to the method of Lowry, the highest absorbance was found between Fraction 6 to 12. The fractionated pEF proteins obtained by gel filtration chromatography were visible by 12% SDS polyacrylamide gel electrophorosis. Molecular weight of 63 kDa band was present in all fractions. Fraction 6 had two more compound of molecular weight of 127 and 97 kDa and fraction 8 had the compound of molecular weight of 127, 97, 86, 76, 35, 25.6, 22 and 20.8 kDa. Molecular masses of fraction 9 were 86, 76, 41, 35 and 25.6 kDa. Fraction 11 had a special 18 kDa band (unpublished data). The fraction 6, 8, 9 and 11 were used for the maturation *in vitro* of porcine immature oocytes.

As shown in Table 5, out of 64 porcine immature oocytes cultured in TCM 199 with intact pEF, 50.0% of the oocytes reached at MII stage. Similar result (48.1%) was obtained when porcine oocytes were cultured in TCM 199 with fraction 11 containing 18 kDa proteins.

DISCUSSION

It is well known that a pivotal function of the epididymis is the production of a luminal environment that promotes maturation and survival of spermatozoa (Syntin *et al.*, 1996). In all mammalian species, sperm originating from the testis need a subsequent phase of subtle transformation that occurs in the epididymis. During sperm journey through this post-testicular organ, sperm acquire motility and fertilization capatibility (Fouchecourt *et al.*, 1999).

In previous study (Yim *et al.*, 2008), lipid component of pEF was removed by treating with n-heptane as des-

Table 4. Effect of porcine epididymal fluid fractions obtained by gel filtration chromatography on *in vitro* maturation of porcine germinal vesicle oocytes

Fractions	No. of oocytes	Maturation stage (%)				
		GV	GVBD	MI	A~T	MII
Control	70	15 (21.4)	30 (43.0)	5 (7.1)	5 (7.1)	15 (21.4)[a]
Intact pEF	64	6 (9.4)	20 (31.2)	4 (6.3)	2 (3.1)	32 (50.0)[b]
Fraction 6	52	7 (13.5)	25 (48.0)	5 (9.6)	4 (7.7)	11 (21.2)[a]
Fraction 8	58	7 (12.1)	30 (51.7)	5 (8.6)	3 (5.2)	13 (22.4)[a]
Fraction 9	56	9 (16.1)	28 (50.0)	5 (8.9)	2 (3.6)	12 (21.4)[a]
Fraction 11	54	4 (7.4)	19 (35.2)	3 (5.6)	2 (3.7)	26 (48.1)[b]

pEF : porcine epididymal fluid, GV: germinal vesicle, GVBD: germinal vesicle breakdown.
MI : metaphase I, A-T : anaphase-telophase, MII : metaphase II.
Column with different letters (a, b) were significantly different ($p<0.05$).

cribed by Byskov *et al.* (1995). No significant difference in the rate of MII stage was observed between n-heptane treatment and intact pEF group. However, the proportion of oocytes remained at GV stage was sinificantly ($p<0.05$) increased in COCs cultured in medium with trypsin treated pEF than those in intact pEF whereas oocytes reached at MII stage was sinificantly ($p<0.05$) increased in COCs cultured in medium with intact pEF than in those with trypsin treated pEF. Those results give clearly the evidence that certain proteins of pEF were capable to promote the nuclear maturation of immature oocytes.

In this study, the rate of metaphase II was significantly ($p<0.05$) increased in oocytes cultured in the media containing cationic protein(s) from pEF compared to those in the media with anionic protein(s) from pEF (Table 2 and 3). And the rate of metaphase II was significantly ($p<0.05$) increased in oocytes cultured in the media containing the fraction 11 obtained by gel filtration chromatography compared to those in the media with other fractions (Table 4). These results suggest that cationic 18 kDa protein(s) from pEF improves the nuclear maturation of porcine immature oocytes. Several groups have shown that the epididymal epithelium synthesizes and secretes numerous proteins, which vary according to epididymal region (Dacheux *et al.*, 1989). Although pEF were collected from same age and region, composition of proteins of pEF from cauda epididymis was very different according to investigators. Métayer *et al.* (2002) reported that pEF from cauda confirmed the presence of the 66, 45, 34~30 and 28 kDa proteins, whereas Dacheux *et al.* (1989) were visualized in protein bands of 135, 125, 96, 84, 63, 60, 51, 23 and 17 kDa. Another report mentioned that pEF from same age and region contain at least major nine molecular weight of 103, 76, 58, 37~40, 31, 28, 21.5, 19 and 16-kDa (Syntin *et al.*, 1996). In present study the major proteins of intact pEF collected from cauda epididymis were composed of at least 11 compounds of molecular mass of 127, 97, 86, 76, 63, 41, 35, 25.6, 22, 20.8 and 18 kDa (unpublished data).

It is not clear whether protein(s) of pEF improves medium milieu or transports directly into oocytes and then activates the signal pathway for meiosis activation.

Significant ($p<0.05$) increase in percentage of MII oocytes was observed when denuded oocytes were cultured in the medium supplemented with intact pEF (Yim *et al.*, 2006). This suggests that the protein(s) of pEF 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 (Legge, 1995), because they still possess cumulus cell projection embedded in the zona pellucida (Hyttel *et al.*, 1986), from which both inhibitory and stimulatory signals may be transferred to the oocytes.

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). 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*. Gonadotrophin or EGF induces maturation of mouse oocytes maintained in meiotic arrest with dbcAMP or hypoxanthine (Downs *et al.*, 1988; Eppig and Downs, 1987). The presence of 1 mM dbcAMP for 20 h (Schultz *et al.*, 1983) and of dbcAMP and Fo (Kim, 2005) completely inhibited the meiotic resumption of porcine cumulus-enclosed ococytes. In agreement with previous studies (Schultz *et al.*, 1983; Kim, 2005), present study showed that most of the porcine immature oocytes cultured in the presence of dbcAMP (88.8%) and forskolin (81.0%) were arrested at the GV stage. Present study also observed that porcine GV oocytes cultured in the medium supplemented with intact pEF appeared to overcome the meiotic arrest caused by dbcAMP and Fo (Table 1).

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. While the cAMP content of intact follicle and 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 gonadotropin 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 is unable to clearly explain the signal pathway of pEF for resumption of meiosis, one explanation for this observation is that oocytes are stimulated to hydrolyze their cAMP by a signal produced by the cumulus cells in response to pEF and/or that a positive factor released from the pEF acts on the oocyte to trigger the resumption of meiosis despite the continued presence of an inhibitor. 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). In addition, IBMX is more effective in preventing the resumption of meiosis in zona-free oocytes than in COCs (Bilodeau *et al.*, 1993).

In coclusion, the present study suggests that 1) dbcAMP and Fo prevent the spontaneous maturation of oocyte after isolation from follicles, and that the inhibitory effect of dbcAMP and Fo can be overcome by intact pEF, 2) cationic 18 kDa protein(s) of pEF are responsible for promotion of MII stage. Further studies

are needed to understand the biochemical characteristics of pEF protein and its cellular mechanism involved in nuclear maturation of mammalian oocytes.

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