Effect of Epididymal Fluid on *In Vitro* Maturation and Subsequent Sperm Penetration in Porcine Follicular Oocytes

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ABSTRACT

The aim of this study is to investigate the effect of porcine epididymal fluid (pEF) on *in vitro*-maturation and subsequent fertilization of porcine follicular oocytes. Porcine cumulus-oocytes complexes retrieved from antral follicles were cultured in tissue culture medium (TCM)-199 supplemented with pEF of different concentrations. At 48 h after culture, development of oocytes to germinal vesicle (GV) breakdown, metaphase I, anaphase-telophase I, and metaphase II were examined. Significant ($p<0.05$) increase in the proportion of oocytes developed to MII stage was observed in oocytes cultured in pEF-containing TCM-199 than in oocytes cultured in pEF-free TCM-199 (46.2% vs 16.7%), which was a dose-dependent manner. Subsequently, the proportion of monospermic fertilization were significantly ($p<0.05$) increased in oocytes cultured in the TCM supplemented with pEF than those cultured in pEF-free TCM-199 (51.0% vs 24.1%). In the second series of experiment, the percentage of MII oocytes was significantly ($p<0.05$) increased after exposure of oocytes to pEF during the first 22 h period of culture than after exposure of oocytes to pEF during the next 24 h of culture, while no significant difference in the percentage of monospermy was observed. The results of this study demonstrate that pEF contains at least enhancing component(s) for nuclear maturation.

(Key words : Porcine, Epididymal fluid, *In vitro* maturation, *In vitro* fertilization, Monospermy)

INTRODUCTION

From fetal to neonatal life, the primary oocytes of mammal are arrested at the diplotene stage of meiosis. Resumption of meiotic division in oocytes normally occurs *in vivo* just prior to ovulation. However, Pincus and Enzmann (1935) showed that rabbit oocytes resume meiosis when they are removed from the ovarian follicles and cultured. Thirty years later, Edwards (1965) reported similar findings for oocytes of pig, cow, and sheep. Maturational (cytoplasmic and nuclear) and developmental (fertilization, pronuclei formation, and cleavage) competencies are influenced by the presence of follicular fluid (FF) (Larocca *et al.*, 1998) and the size of the follicle from which porcine FF (pFF) is harvested (Omran *et al.*, 2004). Maturation media are generally supplemented with protein, such as FBS and BSA (Zheng and Sirard, 1992). Hormonal supplements are achieved by adding various combination of FSH, LH and estradiol. In fact, bovine (Goto *et al.*, 1988) and rabbit (Yoshimura *et al.*, 1989) oocytes matured in the absence of gonadotropins have shown satisfactory developmental competence. Porcine oocytes matured in a medium supplemented with protein and estradiol and without FSH *in vitro* have a poor ability to undergo germinal vesicle breakdown and mature to metaphase II (Nagai *et al.*, 2000). An addition of ejaculated sperm to culture medium has led to 45% metaphase II stage in case of both human (Farhi *et al.*, 1997) and porcine vesicle oocytes (Kim, 2004) even before fertilization, as compared to 10% for spontaneous maturation. The membranes of spermatozoa from adult epididymis have a substance(s) that can enhance *in vitro* maturation of oocytes (Kim *et al.*, 2008). 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 (Fouchecourt *et al.*, 1999). A total of 146 epididymal proteins were found to be secreted by the epididymis. Of the various major proteins, clusterin, glutathione peroxidase, retinol-binding protein, lactoferrin, EP4, beta-N-acetyl-hexosaminidase, alpha-mannosidase, and procathepsin L were identified (Syntin *et al.*, 1996).

The efficiency of producing porcine embryos *in vitro* through IVF of oocytes *in vitro* matured has been improved dramatically in recent years. However, polyspermy remains as a major obstacle to producing large-scale of viable embryos successfully (Day, 2000). Many factors affect penetration of oocytes. Sperm concentration has been shown to be playing a consequ-

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ential role in fertilization in both invertebrates and vertebrates (Yanagimachi, 1981). Amino acid in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated *in vitro* (Ka *et al.*, 1997).

IVM and IVF under a well defined serum-free culture conditions in pig provides a powerful tool for functional evaluation of specific purified proteins and it was hypothesized that those specific proteins might be involved in both *in vitro* maturation and fertilization of porcine follicular oocyes (Kouba *et al.*, 2000).

The objective of this study was to determine whether exposure of oocytes to pEF during *in vitro* maturation improves maturation of oocytes and reduces the rate of polyspermy.

MATERIALS AND METHODS

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

Culture Medium

The basic medium used in this experiment was medium 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 intact porcine epididymal fluid. For IVF of porcine oocytes, modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM Tris-HCl, 11 mM glucose, 5mM sodium pyruvate was used as a basic medium.

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 potassium penicillim-G and 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 ug/ml streptomycin sulfate (pH 7.3) under mineral oil(m 8401; Sigma) in petri dish and cultered in a CO_2 incubator (5% CO_2 in air at 39℃) for 48 h.

Preparation of Epididymal Fluid

The epididymis were obtained at a local slaughterhouse 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.

In Vitro Fertilization

For each experiment, a 0.5 ml straw of ejaculated frozen porcine semen was thawed in a water bath at 37℃ for 1 min. Spermatozoa were washed twice by centrifugation at 1,900 × g for 4 min in Dulbecco's PBS (Gibco, Life Technologies Inc., Grand Island, NY) supplemented with 100 IU/ml potassium penucillin-G, and 50 μg/ml streptomycin sulfate and finally washed in mTBM supplemented with 1 mM caffeine and 0.1% BSA (A-7888). After washing, the final sperm pellet was resuspended in the same medium as used for final washing to give a sperm concentration of 5~6×10^6 spermatozoa/ml. A 50 μl of the final sperm suspension containing 1 mM caffeine and 0.1% BSA was introduced into 50 μl of the mTBM included 10 cumulus-free oocytes for fertilization. The mixture gave final concentrations of 2.5~3×10^6 spermatozoa/ml, 10 mg BSA/ml, and 0.5 mM caffeine and incubated at 39℃ in 5% CO_2 in air with high humidity. After insemination, oocytes were mounted, fixed for 48~72 h in 25% acetic alcohol (v/v) at room temperature, stained with 1% (v/v) orcein in 45% (v/v) acetic acid, and examined for evidence of sperm penetration.

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 hours and then stained with aceto-orcein. Oocytes were observed under phase-contrast optics (×400, Diaphot 300, Nikon, Japan) to classified according to their meiotic stage. Nuclear stage was assorted as germinal vesicle, germinal vesicle breakdown, Metaphase Ⅰ, and Metaphase Ⅱ. Degenerated oocytes were not included in the analysis.

Statistical Analysis

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

RESULTS

Effects of Porcine Epididymal Fluid on *In Vitro* Maturation of Porcine Oocytes

The effects of intact porcine epididymal fluid on *in vitro* maturation of porcine follicular oocytes have shown in Table 1. When porcine immature oocytes were matured in TCM 199 alone (control group), the proportions of oocytes remaining GV stage and reaching MII stage were 34.2% and 16.7%, respectively. The proportion of oocytes reaching MII stage was significantly ($p<0.05$) increased in oocytes cultured in the medium containing pEF compared to those in medium without pEF (46.2% vs 16.7%). However, the proportion of oocytes remaining GV stage were significantly ($p<0.05$) increased in the oocytes cultured in medium without pEF than those in medium with pEF.

Effects of Porcine Epididymal Fluid Concentration

In order to find out the effect of pEF concentration, porcine follicular oocytes were matured in TCM 199 supplemented with different concentration of intact pEF. As shown in Table 2, when porcine immature oocytes were cultured in the TCM 199 supplemented with 2%, 4%, 8%, and 10% pEF, the proportions of oocytes reaching MII stage were 34.3%, 39.0%, 48.9%, and 47.4%, respectively.

Effects of Exposure Period of Porcine Epididymal Fluid

The maturational competence of porcine follicular oocytes exposed to intact pEF at different culture periods has shown in Table 3. When the oocytes were exposed with pEF during first 0~22h culture, the proportion of oocytes reaching MII stage was 47.1%.

Table 1. Effect of porcine intact epididymal fluid on *in vitro* maturation of porcine follicular oocytes.

	No. of oocytes	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
Control	120	41(34.2)	48(40.0)	8(6.7)	3(2.5)	20(16.7)[a]
with pEF	130	18(13.8)	37(28.5)	13(10)	2(1.5)	60(46.2)[b]

GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, A-T: anaphase-telophase, MII: metaphase II.
Values with different superscripts within columns are significantly different (a,b, $p<0.05$).

Table 2. Effect of porcine epididymal fluid concentration on *in vitro* maturation of porcine follicular oocytes

Concentration of epididymal fluid	No. of oocytes	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
Control	94	36 (38.3)	32 (34.0)	5 (5.3)	2 (2.1)	19 (20.2)[a]
2% pEF	102	22 (21.6)	42 (41.2)	0 (0.0)	3 (3.0)	35 (34.3)[b]
4% pEF	100	26 (26.0)	27 (27.0)	2 (2.0)	6 (6.0)	39 (39.0)[b]
8% pEF	92	8 (8.7)	33 (35.9)	5 (5.4)	1 (1.1)	45 (48.9)[c]
10% pEF	97	10 (10.3)	32 (33.0)	5 (5.2)	4 (4.1)	46 (47.4)[c]

pEF: porcine epididymal fluid, GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, A-T: anaphase-telophase, MII: metaphase II.
Values with different superscripts within columns are significantly different (a~c, $p<0.05$).

Table 3. Effect of exposure period of porcine epididymal fluid on *in vitro* maturation of porcine oocytes

Period (h) of exposure	No. of oocytes	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
0~22	104	11(10.6)	32(30.8)	7(6.7)	5(4.8)	49(47.1)[a]
23~44	96	27(28.1)	29(30.2)	2(2.1)	1(1.0)	37(38.5)[b]

GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase, A-T: anaphase-telophase, MII: metphase II.
Values with different superscripts within columns are significantly different (a,b, $p<0.05$).

Table 4. Effect of exposure of porcine epididymal fluid during *in vitro* maturation on subsequent *in vitro* fertilization in porcine follicular oocytes

	No. of oocytes	Penetrated (%)	Mono-spermy	Sperm number / Oocyte (%)†							Average sperm number / oocyte
				2	3	4	5	6	7	8	
Control	83	79 (95.2)	19 (24.1)[a]	12 (15.2)	20 (25.3)	20 (25.3)	1 (1.2)	4 (5.1)	3 (3.8)	0 (0.0)	2.9 (233/79)
with pEF	105	96 (91.4)	49 (51.0)[b]	14 (14.6)	13 (13.5)	10 (10.4)	3 (3.1)	2 (2.1)	3 (3.1)	2 (2.1)	2.3 (217/96)

pEF: porcine epididymal fluid.
Values with different superscripts within columns are significantly different (a,b, $p<0.05$).
† Percentage of the number of oocytes penetrated.

Table 5. Effect of exposure period of porcine epididymal fluid during *in vitro* maturation on subsequent *in vitro* fertilization in porcine follicular oocytes

Period of exposure	No. of oocytes	Penetrated (%)	Mono-spermy	Sperm number / Oocyte (%)†							Average sperm number / oocyte
				2	3	4	5	6	7	8	
0~22	109	102 (93.6)	49 (48.0)	12 (11.8)	7 (6.9)	13 (12.7)	10 (9.8)	6 (5.9)	4 (3.9)	1 (1.0)	2.6 (268/102)
23~44	107	105 (98.1)	47 (44.8)	18 (17.1)	14 (13.3)	10 (9.5)	11 (10.5)	4 (3.8)	0 (0.0)	1 (1.0)	2.4 (252/105)

† Percentage of the number of oocytes penetrated.

The percentage of MII oocytes was significantly ($p<0.05$) decreased in the oocytes exposed at 23~44h period of culture than those in 0~22h culture group.

Effects of Porcine Epididymal Fluid on *In Vitro* Sperm Penetration

The effects of pEF exposure during *in vitro* maturation of porcine immature oocytes on subsequent sperm penetration are shown in Table 4. Even though 95.2% of porcine oocytes matured in TCM 199 without pEF were penetrated by sperm, but only 24.1% of oocytes penetrated were fertilized by single sperm. No significant difference in the percentage of oocytes penetrated was observed both in with/without pEF groups. However, the proportions of oocytes penetrated by single sperm were significantly ($p<0.05$) increased in oocytes matured in medium with pEF than those without pEF.

Effect of Exposure Period of Porcine Epididymal Fluid on *In Vitro* Sperm Penetration

The sperm penetration of porcine oocytes matured in the medium supplemented with intact pEF at different periods has shown in Table 5. When porcine oocytes matured in TCM 199 supplemented with intact pEF at 0~22 h culture period, 93.6% of oocytes were *in vitro* fertilized and 48.0% of oocytes penetrated were fertilized by single sperm penetration. No significant difference in the percentage of monospermic oocytes was observed in both different exposure periods of culture.

DISCUSSION

In all mammalian species, sperm originating from the testis require a subsequent phase of subtle transformations that occur in the epididymis. The composition of the sperm membrane is affected by exposure to the specific intra-luminal environment. Important components in this intra-luminal environment are proteins secreted by the epididymal epithelium. These proteins may change the membrane properties of the spermatozoa in several ways: they may bind to the sperm surface and/or modify the structure or the arrangement of the existing membrane molecules (Vreeburg *et al.*, 1992). In rats and sheep, major glycopeptides present on sperm surface in the terminal part of the epididymis have been found to have an epididymal origin (Voglmayer *et al.*, 1982; Brooks and Tiver, 1984; Zeheb and Orr, 1984). Nuclear maturation of human (Farhi *et al.*, 1997) and porcine (Kim, 2004) germinal vesicle oocytes were significantly enhanced in oocytes co-cultured with human or porcine ejaculated

spermatozoa compared with rate of spontaneous maturation. Previous study has reported that entire plasma membrane of porcine spermatozoa contains a substance(s) that enhances the nuclear maturation prior to fertilization. The putative meiosis enhancing substance(s) present in spermatozoa membrane was acquired from testes after puberty and its biological effect lasts during transportation of spermatozoa through epididymis (Kim *et al.*, 2008). Therefore, I explore the possibility that caudal pEF which take place the final maturation of spermatozoa also contains a meiosis-enhancing substance(s) if ejaculated spermatozoa are able to enhance nuclear maturation of human germinal vesicle oocytes.

Several groups have reported beneficial effects upon maturation of porcine oocytes by using follicular fluid (Vatzias and Hagen, 1999) and in media supplemented with FBS (Zheng and Sirard, 1992), and hormone (Yoshida *et al.*, 1989). The mature follicular fluid collected after LH surge has an adequate level of gonadotropin and steroid hormone (Henault *et al.*, 1995). When serum is added to culture medium, it acts as a source of albumin that balances osmolality and scavengers of harmful molecules (Goud *et al.*, 1998). 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). Pig oocytes can be matured in protein-free medium supplemented with gonadotropins (Abeydeera *et al.*, 1998).

The aim of this study is to evaluate the possible effects of pEF exclusively without any interference from other signaling molecules on maturation *in vitro* of porcine cumulus-oocytes complexes in a chemically defined medium, TCM 199. In this study, the percentage of metaphase II was significantly ($p<0.05$) increased in oocytes cultured in medium containing pEF compared to those in medium without pEF, whereas the proportion of oocytes remaining GV were significantly ($p<0.05$) increased in the oocytes cultured in medium without pEF than those in medium with pEF (Table 1). This study shows that the proportion of oocytes reaching metaphase II was significantly ($p<0.05$) increased from 2% to 10% of pEF (Table 2). This can be interpreted as suggesting that pEF contain a substance(s) that improves the nuclear maturation of oocytes and supplementation of pEF during maturation of oocyte enhances oocytes maturation in a dose-dependent manner *in vitro*. The zona pellucida allows for 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. It is not clear whether a substance(s) from pEF improves medium milieu or transport directly into oocytes and then activates the signal pathway for meiosis activation. The physiological interaction between the oocytes and the beneficial substance(s) from pEF remains to be investigated. It is well known that a pivotal function of the epididymis is the production of a luminal environment that promotes both the maturation and survival of spermatozoa (Syntin *et al.*, 1996). This study is unable to reveal why epithelial cell of male reproductive tract secret meiosis enhancing component(s) for oocytes.

A major problem encountered during fertilization of porcine oocytes *in vitro* is the high incidence of polyspermy. The proportions of monosperm penetration in porcine oocytes were significantly increased in oocytes matured in medium with intact pEF than those without pEF (Table 4, 5). Various attempts to control polyspermy have taken including, pre-incubation of oocytes with oviductal fluid (Dubuc and Sirard, 1995; Kim *et al.*, 1996) and co-culture of oocytes with oviductal epithelial cells and/or conditioned medium (Kano *et al.*, 1994; Dubuc and Sirard, 1995; Vatzias and Hagen, 1999). Porcine oviduct-specific glycoprotein present during IVF reduced the incidence of polyspermy and sperm-zona pellucida binding (Kouba *et al.*, 2000). The conditions controlling the incidence of polyspermy in natural mating have been optimized by evolutionary forces. This is largely achieved through regulating sperm numbers at the site of fertilization within the oviduct. In the pig, the *in vivo* block to polyspermy is thought to result from a restriction of the number of spermatozoa that can reach an egg and the ZP block to penetration (Hunter, 1990). If sperm numbers are increased over those normally found, the number of polyspermic eggs also increases (Hunter, 1973).

Cortical granules are important orgnelles that play a central role in the block to polyspermic penetration during fertilization (Yanagimachi, 1994). After CG exocytosis, CG exudates act on the ZP, causing biochemical and structural changes that cause the ZP to both lose the ability to be penetrated by already-bound spermatozoa and decrease its ability to bind additional sperm (Hao *et al.*, 2006). The migration of CGs to the cortex is a common phenomenon in mammalian oocytes during meiotic maturation not only *in vivo* but also *in vitro* (Yanagimachi, 1994). It is generally believed that the formation of a more or less continuous CG monolayer beneath the plasma membrane is a continuous process until ovulation. In pig oocytes cultured *in vitro*, this migration was almost completed at 26 h after culture, and at this time most of the oocytes had reached M-I (Wang *et al.*, 1997). Delayed cortical granule exocytosis may be the main reason for polyspermy under *in vitro* conditions (Cran and Cheng, 1986). Although this study did not confirm the number of sperm binding to oocyte, one po-

ssible explanation is that the presence of epididymal secretory products in the culture medium creates a unique microenvironment that enhances cortical granule exocytosis during oocyte maturation *in vitro* and reduces polyspermy.

In conclusion, exposure of pEF during maturation of oocyte enhances porcine oocytes nuclear maturation in a dose-dependent manner *in vitro*. This data suggests that pEF contain a substance(s) that improves the nuclear maturation of porcine oocytes. Also supplementation of the maturation medium with pEF reduces polyspermy *in vitro*. However, further studies are needed to identify the mechanism of action of this promoting factor(s) of pEF in order to establish an effective porcine IVM and IVF system.

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