Stimulatory Effect of Porcine Epididymal Fluid on *In Vitro* Maturation of Porcine Immature Oocytes

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ABSTRACT

The aim of this study was to investigate whether addition of porcine epididymal fluid (pEF) into culture medium during *in vitro* maturation influences the nuclear maturation of porcine germinal vesicle (GV) oocytes. Porcine cumulus-oocyte complexes (COCs) from follicles were cultured in tissue culture medium 199 (TCM 199) containing pEF. After 48 hr of 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 (M II) stage was significantly ($p<0.05$) increased in oocytes cultured in the media supplemented with 10% pEF during *in vitro* maturation than in those without pEF regardless of cumulus presence or absence (54.6% vs 22.5%, 51.7% vs 24.2%). The supplementation of pEF during maturation of oocyte enhanced oocytes maturation in a dose-dependent manner *in vitro*. Also significant differences ($p<0.05$) in the percentage of MII oocytes were observed according to exposure period in pEF. Present study suggests that pEF contains a enhancing component(s) for nuclear maturation of porcine immature oocytes *in vitro*.

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

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 fetal calf serum (FCS) and bovine serum albumin (Zheng and Sirard, 1992). Hormonal supplements are achieved by addition of various combinations 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 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. 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).

IVM under defined serum-free culture conditions in pig (Kouba *et al.*, 2000) provides a powerful tool for functional evaluation of specific factors. The objectives of this study were to determine whether exposure of oocytes to pEF during *in vitro* maturation improves maturation of acolytes oocytes.

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 tissue culture medium 199 (TCM 199) supplemented with 100 IU/ml penicillin-G, and 100 μg/ml strepto-

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mycin sulfate (pH 7.3). The medium used in this study was modified by supplementation of intact porcine epididymal fluid.

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 48 hr. In order to examine the effect of pEF on nuclear maturation of cumulus- free oocytes, the cumulus and corona cells were removed from oocytes by treatment of 0.1% hyaluronidase and by repeated passage through a fine pipette.

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.

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. 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 (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

Effect of Intact Porcine Epididymal Fluid

The effect of intact porcine epididymal fluid on *in vitro* maturation of porcine oocytes has shown in Table 1. When porcine immature cumulus-oocytes complexes (COCs) were matured in TCM 199 alone, the proportions of oocytes remained at GV stage and reached at MII stage were 32.7% and 22.5%, respectively. The proportion of oocytes reaching at MII stage was significantly ($p<0.05$) increased in oocytes cultured in the medium containing pEF compared to those in medium without pEF (54.6% vs 22.5%). However, the proportion of oocytes remained at GV stage were significantly ($p<0.05$) increased in the oocytes cultured in medium without pEF than those in medium with pEF. Significant ($p<0.05$) increase in percentage of MII oocytes was also observed when denuded oocytes were cultured in TCM supplemented with pEF.

Concentration of Porcine Epididymal Fluid

In order to find out the effect of pEF concentration on *in vitro* maturation, porcine immature 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 alone, 20.2% of oocytes cultured were able to reach

Table 1. Effect of porcine epididymal fluid on *in vitro* maturation of porcine cumulus-oocytes complex and denuded oocytes in chemically defined medium

Presence or absence of cumulus cell	IVM medium	No. of oocytes	Maturation stage (%)*			
			GV	GVBD	M I	M II
Cumulus +	Non treatment	98	32(32.7)[a]	34(34.7)	10(10.2)	22(22.5)[a]
	10% pEF treatment	97	11(11.3)[b]	22(22.7)	11(11.3)	53(54.6)[b]
Cumulus -	Non treatment	95	28(29.5)[a]	35(36.8)	9(9.5)	23(24.2)[a]
	10% pEF treatment	87	12(13.8)[b]	24(27.5)	6(6.9)	45(51.7)[b]

* GV; Germinal vesicle, GVBD; Germinal vesicle breakdown, M I; metaphase I, M II; metaphase II.
[a,b] Values with different superscripts within columns are significantly different ($p<0.05$).

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

Concentration of pEF	No. of oocytes examined	Maturation stage (%)				
		GV	GVBD	M I	A-T	M II
Control	94	36(38.2)	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(3.1)	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(8.6)	30(34.8)	5(5.4)	1(1.1)	48(52.2)[c]
10% pEF	105	11(10.5)	32(30.5)	7(6.7)	1(0.9)	54(51.4)[c]

* pEF: porcine epididymal fluid, GV; germinal vesicle, GVBD; germinal vesicle break down, MI; metaphase I, MII; metaphase II.
[a-c] Values with different superscripts within columns are significantly different ($p<0.05$).

at MII stage. The proportions of oocytes reaching at MII stage were 34.3% and 39.0% when oocytes were cultured in the medium supplemented with 2% and 4% pEF, respectively. However, significant increase in oocytes reaching at MII stage were observed in oocytes cultured in the medium supplemented with 8% and 10% pEF than in those without pEF.

Exposure Period

The maturational competence of porcine immature oocytes exposed to intact pEF at different periods has shown in Table 3. The proportions of oocytes reaching at MII stage was 50.0%, 51.0% and 52.9% in 0~12 hr, 0~22 hr and 0~44 hr culture group, respectively. No significant difference in the percentage of MII oocytes was observed in three exposure periods of pEF. However, significant decrease in oocytes reaching at MII stage was observed in oocytes exposed at late period of culture (23~44 hr) than in those exposed at early period of culture.

DISCUSSION

In all mammalian species, sperm originating from the testis need a subsequent phase of subtle transformations that occur in the epididymis. The composition of the sperm membrane is changed 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). 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. In rats and sheep, major 17~36 kDa 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). Therefore, we considered that caudal pEF which take place the final maturation of spermatozoa also contains a meiosis-enhancing substance(s) as ejaculated spermatozoa were 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 FCS (Zheng and Sirard, 1992), and hormone (Yoshida

Table 3. Effect of porcine epididymal fluid on *in vitro* maturation of porcine oocytes exposed at different periods of culture

Exposure period (hr)	No. of oocytes examined	Maturation stage (%)*				
		GV	GVBD	M I	A-T	M II
0~12	112	13(11.6)	33(29.5)	6(5.3)	4(3.5)	56(50.0)[a]
0~22	100	10(10.0)	30(30.0)	6(6.0)	3(3.0)	51(51.0)[a]
0~44	104	15(14.4)	30(28.9)	2(1.9)	2(1.9)	55(52.9)[a]
23~44	97	27(27.8)	30(30.9)	2(2.1)	1(1.0)	37(38.1)[b]

* GV; germinal vesicle, GVBD; germinal vesicle break down, MI; metaphase, MII; metaphase II.
[a-b] Values with different superscripts within columns are significantly different ($p<0.05$).

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 design of this study was to evaluate the possible effects of pEF exclusively without 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 MII 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 remained at GV were significantly ($p<0.01$) increased in the oocytes cultured in medium without pEF than those in medium with pEF (Table 1). This study showed that supplementation of pEF during maturation of oocytes enhanced oocytes maturation in a dose-dependent manner *in vitro*. The results of this study suggest that pEF contain a substance(s) that improves the nuclear maturation of oocytes.

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.

In mammals, several culture systems have been developed such as, follicle culture system (Carlos *et al.*, 2000) and oocyte-granulosa cell complex culture system (Byskov *et al.*, 1995), which allows growing oocytes. However, it is difficult to investigate which factors affect the *in vitro* maturation of porcine oocytes. Several authors have reported the evidence suggesting that porcine oocytes matured in protein-free medium supplementation with steroid hormone (Vatzias and Hagen, 1999) and sera that have been used for *in vitro* fertilization. Previous reports showed that supplementation of maturation media with follicular fluid promoted maturation and further development of porcine oocytes (Naito *et al.*, 1988). A total of 146 epididymal proteins were secreted by the epididymis. Of the various major proteins, clusterin, glutathione peroxidase, retinol-binding protein, lactoferrin, EP4, beta-N-acetylhexosaminidase, alpha-mannosidase, and procathepsin L were identified (Syntin *et al.*, 1996). However, the pEF digested by trypsin were not able to improve the rate of nuclear maturation (unpublished data). 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.

However, it is difficult to clarify which factor(s) can affect the maturation of oocytes by pEF because many unknown factors are present in pEF. The exact biochemical characteristic of beneficial substance(s) from pEF 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 culture system for *in vitro* production of porcine embryos.

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