# Effect of Porcine Epididymal Fluid on *In Vitro* Maturation of Porcine Germinal Vesicle Oocyte

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## ABSTRACT

**The aim of this study was to investigate what components of porcine epididymal fluid (pEF) influences the nuclear maturation of porcine germinal vesicle oocytes. Porcine cumulus-oocytes complexes from follicles were cultured in TCM 199 containing pEF. After 48 h cultures, oocytes were examined for evidence of GV breakdown, metaphase I, anaphase-telophase I, and metaphase II. Maturation rate of oocytes was significantly increased in media supplemented with 10% pEF during *in vitro* maturation (IVM) than in those without pEF. When lipid component of pEF was removed by treating n-heptane, no significant difference was observed in maturation of oocytes between n-heptane treatment and intact pEF group. However, the proportion of oocytes reaching at metaphase II (M II) was significantly ($p$<0.05) decreased in the oocytes cultured in media containing trypsin-treated pEF compared to those in media with intact pEF. When porcine GV oocytes were matured in the medium supplemented with intact pEF or pEF heated at 56℃ and 97℃, rates of oocytes remained at GV stage were 11.7%, 29.4% and 42.0%, respectively. However, there were no difference in proportion of oocytes reaching at MII stage among intact pEF group and 56℃ group.**

**Present study suggests that 1) pEF contains an enhancing component(s) for nuclear maturation *in vitro* of oocytes, 2) protein(s) of pEF may be capable to promote nuclear maturation *in vitro*, and 3) enhancing component for nuclear maturation may consist of two factors, which are responsible for germinal vesicle breakdown (GVBD) and promotion of MII stage.**

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

## INTRODUCTION

Mammalian oocytes are arrested at the prophase of the first meiotic cycle before 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). Several authors have reported evidence suggesting that maturation of germinal vesicle oocytes was enhanced by the addition of various gonadotropin (Yoshimura *et al.*, 1989; Goto *et al.*, 1998) and steroid (Nagai *et al.*, 2000). After 44 hours of culture, the maturation rate was significantly higher($p$<0.01) in LH-(76%) and FSH-(86%) treated oocyte than in the non-treatment control oocytes (35%). Farhi *et al.* (1997) observed that compared with the 10% rate of spontaneous maturation, addition of ejaculated sperm to culture medium led to metaphase II in 45% of human germinal vesicle oocytes even if before fertilization. We have reported that the membrane of spermatozoa from adult epididymis have a substance(s) that can enhance IVM 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 (Antczak *et al.*, 1997). It is likely that changes in the composition of the sperm membrane are induced by exposure to the specific intraluminal enviroment. Important components in this intraluminal enviroment 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* (Yim *et al.*, 2006). The exact biochemical characteristic of beneficial substance(s) from pEF remains to be clarified. The present study was performed to test wh-

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at components of pEF were able to stimulate porcine oocytes maturation.

## MATERIALS AND METHODS

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

### Preparation of Porcine Epididymal Fluid

Adult porcine epididymis were obtained from freshly killed animals at the local slaughterhouse and transported to the laboratory within 1 h in 0.85% (w:v) NaCl solution at 4℃. pEF was aspirated from cauda, which take place the final maturation of spermatozoa. Spermatozoa were separated from the fluid by centrifugation (15 min, 15,000×g) and supernatant epididymal fluid was used directly or stored at −80℃ with same volume glycerol.

### Preparation of Oocyte and IVM

Porcine ovary were obtained at a local slaughterhouse and transported to the laboratory within 1 h in 0.85% (w:v) NaCl solution at 30℃. Random breed porcine oocytes were aspirated from follicles 3~5 mm in diameter with an 18-gauge needle fixed to a 10-ml disposable syringe. Aspirated oocytes were collected in 10-ml test tube. The supernatant was discarded from test tube when sediment becomes visible. The basic medium used for the maturation of oocytes was culture medium TCM-199 (with Earle's salts; Gibco, Grand Island, NY). Collected oocytes were washed three times with maturation medium and transferred to culture dish under mineral oil in a polyethylene culture dish. The dishes were cultured for 24 h under 5% $CO_2$ in air at 39℃.

### 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 cover glass 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 classify 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.

## Experiment Design

### Experiment 1. Effects of pEF on IVM of porcine cumulus oocyte complexes (COCs)

To evaluate effect of pEF on the progress of nuclear maturation of porcine oocytes, COCs were cultured in medium supplemented with 10% pEF. After 24 h of culture with pEF, oocytes were washed three times with TCM-199. They were than transferred in to 100 ul of the same medium without pEF and cultured for 24 h under 5% $CO_2$ in air at 39℃.

### Experiment 2. Effects of n-heptane treatment of pEF on IVM of porcine COCs

To test whether lipid component of pEF can or not enhance the resumption of COCs, lipid component of pEF was removed by treating with n-heptane as described by Byskov *et al* (1995). Briefly, pEF was centrifuged at 15,000g for 15 min. A 20 ml portion of pEF was treated with 40 ml n-heptane in the dark for 15 h. The organic phase was discarded.

During first 24 h culture COCs were cultured in the medium supplemented with n-heptane-treated pEF.

### Experiment 3. Effects of trypsin treatment of pEF on IVM of porcine COCs

To test the hypothesis that protein components of pEF are capable to induce meiosis resumption of COCs, proteins of pEF were digested by treatment of trypsin. Aliquots of solution containing pEF were incubated with trypsin (T4799) solution (0.1 ug/ul) in 37℃ water bath for 2 hours. The reactions were stopped by placing the tubes on ice. Digestion of pEF protein was confirmed by gel electrophoresis.

During first 24 h culture COCs were cultured in the medium supplemented with trypsin-treated pEF.

### Experiment 4. Effect of Heat Treatment of pEF on IVM of porcine COCs

To evaluate heat stability of meiosis enhancing components of pEF, pEF was placed in a clean test tube, covered with aluminum foil and heated at 56℃ water bath for 30min or 97℃ water bath for 15 min. During first 24 h culture COCs were cultured in the medium supplemented with heat-treated pEF.

### Statistical Analysis

Statistical analysis was performed with a standard computerized statistics program using $\chi^2$.

A probability of $p<0.05$ was considered statistically significant.

## RESULTS

### Effect of Intact Porcine Epididymal Fluid

The effect of intact pEF on IVM of porcine oocytes has shown in Table 1. When porcine COCs were ma-

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

| Presence or absence of pEF | Total no. of germinal vesicle oocytes | Maturation stages of oocytes (%) | | | |
|---|---|---|---|---|---|
| | | GV | GVBD | M I | M II |
| 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] |

pEF : porcine eididymal fluid, GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, M I : Metaphase I, M II: Metaphase II.
Values with different superscripts within columns were significantly different ([a,b], $p<0.05$).

**Table 2. Effects of n-heptane treatment of porcine epodidymal fluid on maturation of porcine cumulus-oocytes complex *in vitro***

| Treatment | Total no. of examined oocytes | Stages of oocytes after maturation (%) | | | |
|---|---|---|---|---|---|
| | | GV | GVBD | M I | M II |
| Intact pEF | 100 | 11(11.0) | 23(23.0) | 11(11.0) | 55(55.0) |
| n-heptane treated pEF | 99 | 10(10.1) | 24(24.5) | 8(8.1) | 57(57.6) |

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, M I : Metaphase I, M II: Metaphase II.

tured 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.

### Effects of n-Heptane Treatment of pEF

To removal of lipid from pEF, pEF was treated with n-heptane. As shown in Table 3, in n-heptane treatment group the proportion of oocytes reaching at M II stage was 57.6%. No significant difference in the rate of M II stage was observed between n-heptane treatment and intact pEF group.

### Effects of Trypsin Treatment of pEF

After aliquots of solution containing pEF were incubated with trypsin in 37℃ water bath for 2 hours, the maturational competence of COCs is shown in Table 3. The rate of M II stage in intact control group was 57.5%, whereas 15.9% of COCs cultured in medium with trypsin-treated pEF reached at MII stage. However, the proportion of GV oocytes was significantly increased in COCs cultured in medium with trypsin-treated pEF than those in intact control.

### Effect of Heat Treatment of pEF

The maturation stage of porcine COCs according to heat treatment of pEF is shown in Table 4. Of 103 COCs matured in TCM with intact pEF, 54.4% (56/103) reached at MII stage. When COCs were cultured in TCM 199 with pEF treated at 56℃ or 97℃, the proportion of COCs reaching at MII satge was significantly decreased than those of intact control group. But the proportion of COCs remained at GV stage was significantly increased in COCs of pEF treated at 56℃ or 97℃ group than those of intact control group.

## DISCUSSION

Several groups have reported beneficial effects upon

**Table 3. Effects of trypsin treatment of porcine epodidymal fluid on maturation of *in vitro* porcine cumulus-oocyte complex in chemically defined medium**

| Treatment | Total no. of examined oocytes | Stages of oocytes after maturation (%) | | | |
|---|---|---|---|---|---|
| | | GV | GVBD | M I | M II |
| Intact pEF | 87 | 8(9.2)[a] | 24(27.6) | 5(5.7) | 50(57.5)[a] |
| Trypsin treatment | 88 | 34(38.6)[b] | 32(36.4) | 8(9.1) | 14(15.9)[b] |

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, M I : Metaphase I, M II: Metaphase II.
Values with different superscripts within columns were significantly different ([a,b], $p<0.05$).

**Table 4. Effect of heating treatmented of porcine epididymal fluid on of *in vitro* maturation porcine cumulus-oocyte complex**

| Heating | Total no. of oocytes | Stages of oocytes after maturation (%) | | | |
|---|---|---|---|---|---|
| | | GV | GVBD | M I | M II |
| Intact | 103 | 12(11.7)[a] | 22(22.3) | 12(11.7) | 56(54.4)[a] |
| 56℃ treatment 10% pEF | 204 | 60(29.4)[b] | 40(19.6) | 6(2.9) | 98(48.0)[a] |
| 97℃ treatment 10% pEF | 250 | 105(42.0)[c] | 43(16.8) | 5(2.0) | 98(39.2)[b] |

GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, M I: Metaphase I, M II: Metaphase II.
Values with different superscripts within columns were significantly different ([a~c], $p<0.05$).

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 *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). Because many factors are present in the cumulus cell mass, follicular fluid and FCS, it is difficult to investigate which factors affect the IVM of oocytes. 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.

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). In all mammalian species, sperm originating from the testis need a subsequent phase of subtle transformations that occur in the epididymis. During their journey through this posttesticular organ, sperm acquire motility and fertilization capability (Fouchecourt *et al.*, 1999). Nuclear maturation of human germinal vesicle oocytes was significantly enhanced in oocytes co-cultured with human ejaculated spermatozoa compared with rate of spontaneous maturation (Farhi *et al.*, 1997). Similarities between epididymal fluid proteins and sperm surface compounds were reported by Russell *et al.* (1984) and Dacheux *et al.* (1989). Previous study has reported that caudal pEF which take place the final maturation of spermatozoa contains a meiosis-enhancing substance(s) (Yim *et al.*, 2006).

In this study, the rate of metaphase II was significantly ($p<0.05$) increased in oocytes cultured in media containing pEF compared to those in media without pEF, whereas the proportion of COCs remained at GV were significantly ($p<0.05$) increased in the oocytes cultured in media without pEF than those in media with pEF (Table 1). The results of this study suggest that pEF contain a substance(s) that improves the rate of nuclear maturation.

In mammals two meiosis activating sterols (MAS) have been found to activate meiotic resumption in mouse oocytes *in vitro* (Byskov *et al.*, 1995). In mouse oocytes arrested with hypoxanthine, both T (testicular)-MAS and FF (follicular fluid)-MAS overcome the inhibitory effect of hypoxanthine and induce resumption of meiosis in dose-dependent way (Fouchecourt *et al.*, 1999). T-MAS has been measured in concentration of around 30ug/g testes-tissue of adult bull, horse, man, mouse, and rat (Byscov *et al.*, 1999). We considered that T-MAS from testes plays the important role for initiating meiosis of oocyte. However, no significant difference in the rate of M II stage was observed between n-heptane treatment and intact pEF group. This result suggests that meiosis-enhancing substance in epididymal fluid is not lipid or cholesterol. However, the rate of M II stage was significantly decreased in COCs cultured in medium with trypsin-treated pEF than those in intact fluid (Table 3). The present study gives clearly the evidence that certain protein(s) of pEF were able to improve the rate of nuclear maturation. The maturation promoting protein of pEF is evident for cumulus-free oocytes also (Yim *et al.*, 2006), suggesting that the protein 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 proportion of COCs reaching at MII stage was significantly decreased in COCs of pEF treated at 97℃ group than those of intact control group, whereas the

proportion of COCs remained at GV stage was significantly increased in COCs of pEF treated at 56℃ or 97℃ group than those of intact control group (Table 4). However, no differences were observed in maturational stage of oocytes among intact, 56℃ and 97℃ treat groups when the values of oocytes remained at GV stage were omitted from Table 4. Although the proportion of COCs remained at GVBD stage was tend to increase in the oocytes cultured in media without pEF than those in media with pEF, the rate of metaphase II was significantly ($p$<0.05) increased in oocytes cultured in media containing pEF compared to those without pEF (Table 1). From those results, since large number of oocytes in heat-treated pEF group or in no pEF group was still remained at GV or GVBD stage, respectively, rate of MII stage may be deceased. This data suggest that protein of pEF for GVBD is destroyed by treatment of heat and that another factor responsible for progression from GVBD to MII stage is stable to heat.

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. Mėtayer *et al.* (2002) reported that pEF from cauda confirmed the presence of the 66, 45, 34~30 and 28kDa proteins, whereas Dacheux *et al.* (1989) were visualized in protein bands of 135, 125, 96, 84, 63, 60, 51, 23 and 17kDa (Dacheux *et al.*, 1989). 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 16kDa (Syntin *et al.*, 1996). Although molecular mass of major band of pEF treated at 56℃ was similar to intact pEF (Unpublished data), the proportion of COCs remained at GV stage was significantly increased in COCs of pEF treated at 56℃ or 97℃ group than those of intact control group (Table 4). This result suggests that certain protein of intact pEF is responsible for GVBD. In 97℃ heat treatment of pEF, meiosis-activating activity responsible for progression from GVBD to MII stage was still conserved. It is not clear whether intact and tranformed protein of pEF improves medium milieu or transports directly into oocytes and then activates the signal pathway for meiosis activation.

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 oocytes culture system.

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