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Addition of Oviductal Fluid to the Fertilization Medium Enhances Monospermic Penetration and Subsequent *In Vitro* Development of Porcine Oocytes

Kim, N. H., S. J. Moon*, J. G. Lim, D. B. Koo, H. T. Lee and K. S. Chung

Animal Resources Research Center, Academy of Life Science,
Kon Kuk University, Seoul 133-701, Korea

체외수정시 배양액내 난관액 첨가가 돼지 난포란의 수정율 및 배 발달율에 미치는 영향

김남형 · 문승주* · 임준교 · 구덕본 · 이훈택 · 정길생

건국대학교 동물자원연구센터

요 약

돼지난포란의 체외성숙, 체외수정 및 체외 배양체계의 개발은 핵치환에 의한 복제동물 및 형질전환 동물생산 등과 같은 첨단생명공학연구 추진에 크게 기여할 것이다. 그러나 돼지난자의 체외수정시 다정자 침입은 큰 문제점으로 지적되고 있는데, 그 원인 및 개선책은 많은 연구에도 불구하고 정확히 밝혀져 있지 않다. 따라서 본 연구는 돼지 난포란으로부터 채취한 난자를 체외성숙 후, 체외수정시 체외수정 배양액에 난관액을 첨가하여 수정율 및 다정자 침입율을 조사하고, 또한 체외수정 후 체외 배 발달율을 조사할 목적으로 실시하였다. 수정용 배양액에 1와 5% 난관액을 첨가하였을 때 정자침입율과 수정시 난자에 침입한 정자의 평균 수가 감소하였으며, 또한 투명대에 부착된 정자의 수도 감소하였다. 난관액이 첨가된 수정용 배양액에서 정자를 1.5와 3 시간동안 전 배양을 실시하였을 때 정자의 수정능 획득과 첨체반응이 대조군에 비해 증가되었다. 그리고 1% 난관액이 포함된 배양액에서 체외수정된 난자의 배반포까지 발달율은 18.9%로 대조구의 12.4%보다 유의성 있게 높았다. 이러한 연구결과는 난관 분비액의 어떤 인자가 정자의 첨체반응을 유도해서 정자침입율 및 다정자 침입을 감소시키고, 이어 배발달율을 증가시킨다고 사료된다.

I. INTRODUCTION

There are many recent reports describing the successful *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of pig oocytes. However, the abnormally higher incidence of polyspermy is one of the major problems following IVM and IVF in the pig (Nagai et al., 1984; Yodhida et al., 1993; Funahashi and Day, 1993; Kim et al., 1996a). Addition of porcine oviductal epithelial cells has been used to reduce the incidence of polyspermy in porcine oocytes (Nagai and Moor, 1989). Nagai and Moor (1989) have suggested that macromolecules secreted from oviductal cells reduced polyspermy in porcine oocytes fertilized *in vitro* by means of some interaction with fertilizing sperm. Zona-binding of glyco-

* 전남대학교 낙농학과(Dept. of Dairy Science, Chun Nam University)

proteins of boar spermatozoa with zona pellucida glycoproteins is inhibited by sulfated polymers, such as dextrone sulfate, polyvinylsulphate and heparin (Jones, 1991; Parry et al., 1992). Therefore, glycoproteins (probably sulfated form) in oviductal fluid may reduce the attachment of spermatozoa to the zona pellucida, which reduces penetration and polyspermy.

Under *in vivo* condition, oocytes are always bathed in an environment of oviductal fluid before fertilization. Parrish et al. (1989) have examined the role of oviductal fluid for the capacitation of bovine spermatozoa. Their results suggest that a heparin-like glycosaminoglycan from the oviduct may be an active component for capacitation in the oviduct. Lack of evidence is available on the effects of oviductal secretion on the *in vitro* fertilization in the pig. In this study we examined the effects of oviductal fluid on the sperm penetrability and subsequent development *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. Preparation of oviductal fluid

Oviductal fluid was collected from cycling gilts as described by Archibong et al. (1989). Briefly, gilts were anesthetized at day 17 to 18 of the estrous cycle. A plastic cannula (I.D., 1.25 mm; O.D., 2.25 mm) was inserted into each oviduct and ligated at the isthmus, approximately 6 mm from the utero-tubal junction to prevent accumulated fluid from flowing into the uterus. The distal end of each cannula was inserted through the cap of a 10 ml conical tube and secured in situ with medical grade silicone adhesive. Oviductal fluid was collected at 12 h intervals for 5 days. The fluid from days 20 and 21 of the estrous cycle was used in this experiment. The fluid was centrifuged at 1,000g for 10 min. and the supernatant was filtered and frozen at −20 ℃ until used.

2. *In vitro* maturation

Porcine oocyte-cumulus complexes (OCC) with uniform ooplasm and a compact cumulus cell mass were prepared in HEPES buffered TALP medium containing 0.1% polyvinylalcohol (H-TL-PVA, 7). Culture medium for *in vitro* maturation was BSA-free NCSU23 (Petters and Wall, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 10 IU/ml eCG and 10 IU/ml hCG. Fifty OCC were transferred to 500 ml of NCSU23, covered with paraffin oil in a four well culture plate, and then cultured for 44 h at 39℃ in an atmosphere of 5% CO_2 in air.

3. *In vitro* fertilization

Sperm-rich fraction (15ml) was collected from a boar by the gloved-hand method, and kept at 20℃ for 16h after adding an antibiotic-antimycotic solution (Gibco). The semen was washed 3 times with 0.9% (w/v) NaCl supplemented with 1 mg/ml BSA (Fraction V, Sigma) by centrifugation. The sperm suspension was incubated for 90 min. at 39℃ in an atmosphere of 5% CO_2 in air. Ten oocytes were washed three times with Tris buffered medium supplemented with 10 mM caffeine sodium benzoate and 4 mg/ml BSA at pH 7.4 and placed into a 50 ml droplet of the mM199 under paraffin oil. Fifty microliter of diluted preincubated sperm was added to 50 ml of the medium containing oocytes so that a final sperm concentration of 5×10^5 cells/ml was obtained. Oocytes were co-cultured with spermatozoa for 6 h at 39℃ in an atmosphere of 5% CO_2 in air. The oocytes were then transferred to 500 ml of fresh NCSU23 and cultured at 39℃ in an atmosphere of 5% CO_2 in air.

4. Chlorotetracycline (CTC) assessment of spermatozoa

The methods for CTC assessment were essentially the same as those described by Wang et al. (1991). Briefly, the sperm suspension was incubated for 3 min. at room temperature in the dark, layered onto 4 ml of 3% (w/v) polyvinylpyrrolidone (PVP, Sigma) in PBS and centrifuged at 500 g for 6 min. The spermatozoon pellet were resuspended in 50 ml of the tris buffer medium. From this suspension, 45 ml was added to 45 ml CTC solution, and after mixing, 8 ml of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris-HCl l-1 (pH 7.4) was added with mixing. The CTC solution was prepared daily by adding 750 mM CTC l-1 (Sigma) and 5 mmol cysteine l-1 (Sigma) to a buffer containing 130 mM NaCl and 20 mM Tris (Trizma base: Sigma); the pH was adjusted to 7.8. Slides were prepared by placing 10 ml of the fixed sperm suspension on a slide and one drop of 0.22 mol 1,4-diazabicycloctane l-1 (Sigma) dissolved in glycerol : PBS (9:1) was mixed in carefully to retared the fading of fluorescence. The spermatozoa were assessed under phase contrast microscope (Nikon, Japan) equipped with epifluorescenct optics on the same day.

5. Assessment of sperm penetration

At the end of culture, eggs were fixed at least 48 h in 25% (v/v) acetic alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined for sperm penetration under a phase contrast microscope at a magnification of ×200 and ×400. Oocytes were designated as penetrated when they had at least one sperm nucleus or male pronucleus with corresponding sperm tail in the vitellus. Those oocytes with more than one sperm nucleus or male pronucleus were considered to be polyspermic.

6. Statistical analysis

Statistical analyses of data from three replicate trials were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test. The percentage data were subjected to arcsine transformation before statistical analysis. The mean number of spermatozoa penetrated to the zona was expressed as mean (SEM. Probability of $P<0.05$ was considered to be statistically significant).

Ⅲ. RESULTS AND DISCUSSION

Supplementation of oviductal fluid to the fertilization medium decreased penetration rate and the mean number of spermatozoa in penetrated eggs as compared with control (Fig. 1). Percentages of monospermic oocytes were also higher when oviductal fluids were added to the fertilization medium. When oocytes were inseminated in medium containing either oviductal fluid, the number of spermatozoa tightly binding on the zona pellucida was less than that in the control group (Fig. 2). Previously, Day and Polge (1968) observed polyspermic penetration of pig oocytes *in vivo* following induced relaxation of uterotubal junction by progesterone injection. Results suggest that the presence of an abnormally large number of spermatozoa at egg surface may be a factor of polyspermy during *in vitro* fertilization in the pig. Although the exact mechanism by which the oviductal cells or oviductal fluid modulate the number of spermatozoa reaching to the eggs *in vitro* is not established, it has been suggested that oviductal cells or its secretion regulate the number of spermatozoa with fertilizable ability that reach the site of fertilization in the pig (Hunter, 1991; 1994, Kim et al., 1996c). Nagai and Moor (1989) reported that the addition of porcine oviductal cells to the fertilization medium reduced polyspermy in porcine oocytes fertilized *in vitro* by means of some interaction with fertilizing sperm. Recently, Dub-

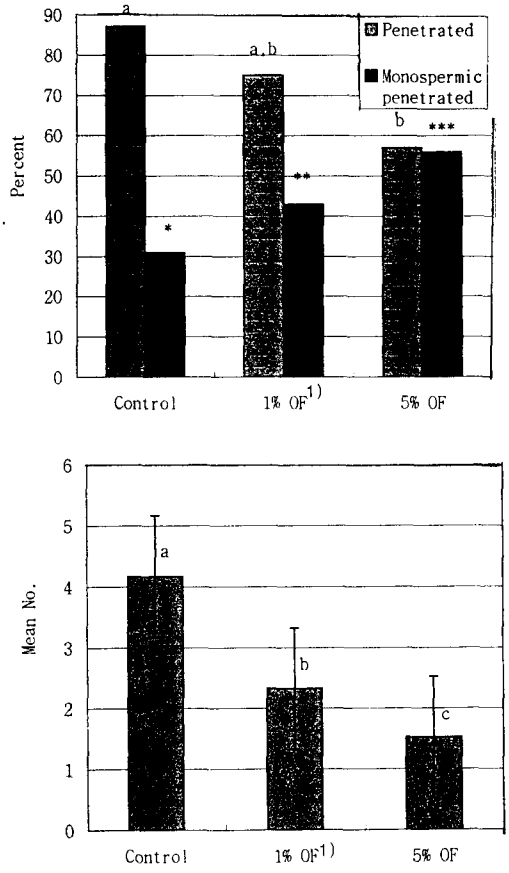

Fig. 1. Effect of oviductal fluid in *in vitro* fertilization medium on sperm penetration of porcine oocytes.

a,b,c: *,,*** Different superscripts within columns denote significant differences ($P<0.05$).**

1) Oviductal fluid.

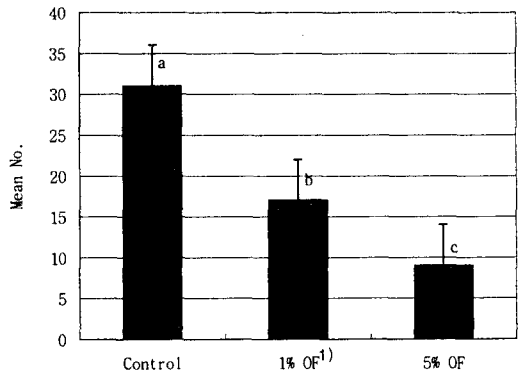

Fig. 2. Number of spematozoa that attached firmly to the zona surface of pig oocytes in presence with oviductal fluid in the fertilization media.

a,b,c Different superscripts within columns denote significant differences ($P<0.05$).

1) Oviductal fluid.

uc and Sirard (1995) cocultured boar spermatozoa with oviductal cells for 30 minutes, so that the spermatozoa could bind to the oviductal cells. The spermatozoa that were bound to the oviductal cells were capable of fertilizing the eggs and the nonbound spermatozoa had a reduced penetration incidence.

The number of spermatozoa firmly attached to the zona pellucida at 12 h after insemination decreased in the presence of oviductal fluid. It therefore appears that the exposure of spermatozoa to oviductal fluid reduced the number of spermatozoa reaching and binding to the zona pellucida which may reduce polyspermy. It has been known that only acrosome reacted spermatozoa can penetrate the zona pellucida in the guinea pig (Huang, 1981). In contrast, acrosome-intact spermatozoa, not acrosome reacted, bind to the zona pellucida in the mouse (Schtoeder, 1991). Since the zona pellucida glycoproteins of porcine oocytes are similar to those of mouse oocytes (Wassarman, 1988), the interaction between spermatozoa and zona pellucida may therefore be similar to those in mice. Previously, Funahashi and Day (1993) reported that prefertilization incubation of spermatozoa in the presence of porcine follicular fluid induced the acrosome reaction of spermatozoa and decreased attachment of spermatozoa to the zona pellucida

Table 1. Effect of oviductal fluid of in the fertilization media on *in vitro* developments of porcine oocytes following insemination

Potassiumc ocentration	No. of embryos examined	No. (%) of embryos degenerated	No. (%) of embryos that developed to			
			1 cell	2 to 8 cell	Morula	Blastocyst
Control	226	10 (4.4)	20 (8.8)	109 (48.2)	59 (26.1)	28 (12.4)
1% OF	217	16 (7.4)	14 (6.5)	75 (34.6)	71 (32.7)	41 (18.9)*
5% OF	202	11 (5.4)	42 (20.8)	82 (40.6)	45 (22.2)	22 (10.9)

with a subsequent increase in the monospermic penetration. Therefore, the component(s) of oviductal fluid may enhance spontaneous acrosome reaction of boar spermatozoa which may result in the reduction of attachment of spermatozoa to the zona pellucida followed by increase of incidence of monospermic penetration.

The time course of incubation of boar spermatozoa in the presence of 5% oviductal fluid is shown in Table 1. When spermatozoa were cultured with either oviductal fluid for 1.5 and 3 h, the incidence of spermatozoa with AR pattern was significantly higher than that of controls (Fig. 3A). Incubation of spermatozoa with oviductal fluid for a period of 1.5 h and 3 h increased the percentage of spermatozoa with pattern B (Fig. 3B). CTC staining has been used to assess the functional status of the mouse (Ward and Storey, 1984), human (DasGupta, 1993), bull (Fraser, 1995) and boar spermatozoa (1995). The major advantage of CTC staining is that it not only allows discrimination between acrosome-intact and acrosome reacted spermatozoa, but also divide acrosome-intact cells into uncapacitated and capacitated groups (Fig. 4). The present study demonstrated that incubation of spermatozoa in the presence of oviductal fluid increased the capacitation and acrosome reaction but decreased attachment of spermatozoa to the zona pellucida with subsequent increase in monospermic penetration. These results suggest that oviductal secretions increase the rates of monospermic entry by induction of the acrosome reaction and a consequent reduction in number of spermatozoa that bind to the zona

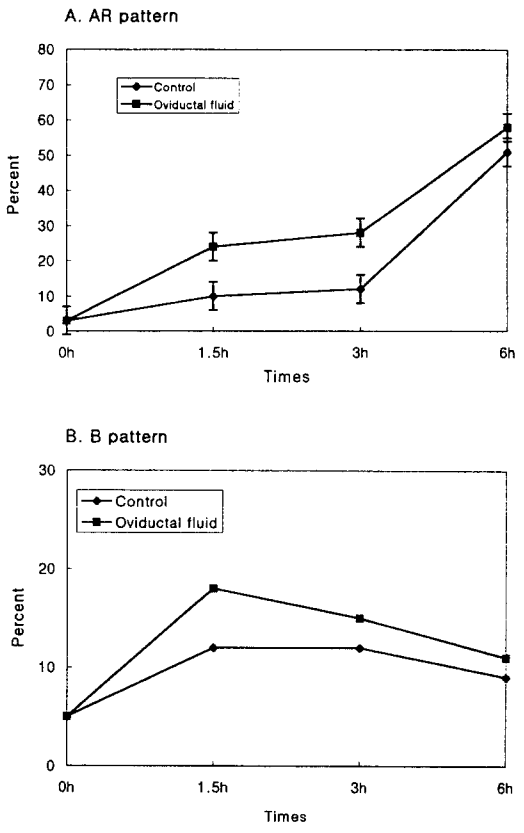

Fig. 3. Changes in chlortetracycline (CTC) fluorescent patterns in boar spermatozoa incubated for 6h in tris buffered medium in the absence or presence of 5% oviductal fluid and sampled at various intervals. Each point represent the mean (SE) of three trials (* $P<0.05$).

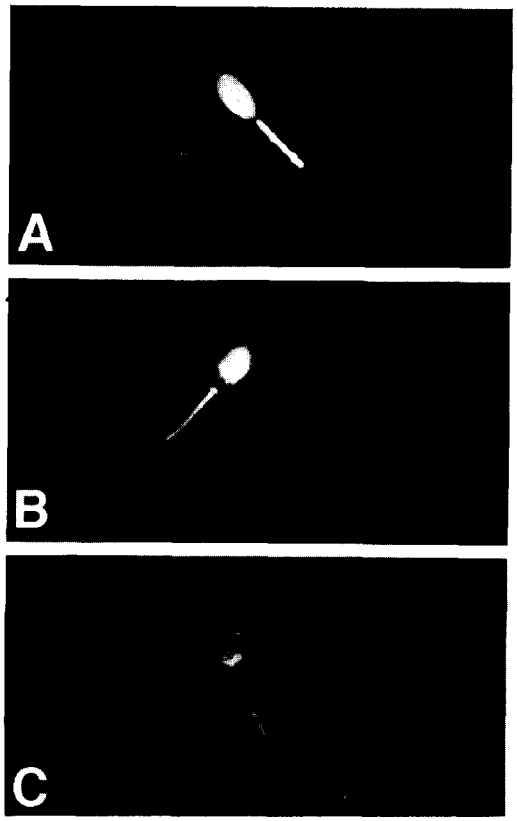

Fig. 4. Three patterns of chlortetracycline fluorescent staining observed on boar spermatozoa cultured for 6h in a tris buffered medium.
(A) F, with uniform fluorescence over the whole head; (B) B, with a fluorescence-free band in the post acrosomal region; (C) AR, with dull fluorescence over the whole head except for the thin and bright band of fluorescence in the equatorial segment.

pellucida.

Polyspermy has been one of the major problems for the successful IVF in the pig. Although the rate of monospermy and mean number of spermatozoa were improved in this study with use of oviductal fluid, the rate of polyspermy is still high. As shown in the Table 1, the percentage of IVM /IVF derived embryos with 1% oviductal fluid that developed to the blastocyst (Fig. 5) was significantly higher than that in control. This result is well accord with the result by the Funahashi et al. (1994) who reported that developmental ability of porcine embryos is reduced by the existence of accessory spermatozoa in the cytoplasm, even if only one male and female pronucleus were formed after fertilization *in vitro*.

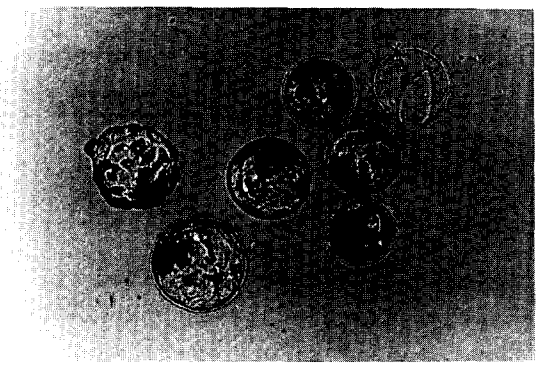

Fig. 5. Porcine blastocysts developed *in vitro* by IVM-IVF.

In conclusion, these results suggested that the factor(s) in the secretion from the oviduct reduce polyspermic fertilization and the number of spermatozoa that penetrate porcine oocytes. The reduction of polyspermic penetration by oviductal secretion may be due to induction of capacitation and acrosome reaction. Further experiments are needed to determine the precise mechanism underly oviductal secretions on the reduce incidence of polyspermic penetration in pig oocytes.

Ⅳ. SUMMARY

The objective of this study was to determine effects of oviductal fluid on the sperm penetration and subsequent *in vitro* development of

porcine oocytes. The addition of oviductal fluid to the fertilization medium decreased sperm penetration and the mean number of spermatozoa in penetrated eggs. The number of spermatozoa firmly bound to zona pellucida was also decreased in the presence of oviductal fluid. Chlortetracycline (CTC) fluorescence patterns were used to determine incidence of capacitation and acrosome reaction. The proportion of capacitated and acrosome free spermatozoa increased when spermatozoa were exposed for 1.5 and 3 h to oviductal fluid. These results suggest that the factor(s) in secretion from the oviduct reduces polyspermic fertilization and the number of spermatozoa that will penetrate porcine oocytes. The reduction of polyspermic penetration by oviductal secretions may be due to a reduced number of spermatozoa in the fertilization medium into an intact acrosome.

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