

Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes *in vitro*

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The effects of oviductal fluid on sperm penetration and cortical granule exocytosis in pigs were examined. Cortical granule exocytosis in oocytes matured *in vivo* and *in vitro* was observed by staining with fluorescent-labelled lectin and laser-scanning confocal microscopy. Exocytosis of matured oocytes was classified into three categories after *in vitro* fertilization: complete cortical granule exocytosis and even distribution of exudate in the entire perivitelline space (type I); complete exocytosis and partial distribution of exudate (type II) and incomplete cortical granule exocytosis (type III). The incidence of oocytes with type I exocytosis was higher in oocytes matured *in vivo* than in those matured *in vitro*. The addition of oviductal fluid at a concentration of 1% or 10% to the fertilization medium decreased sperm penetration and the mean number of spermatozoa present in penetrated eggs. The distribution of cortical granule exudate was not different in the presence of 1% oviductal fluid after sperm penetration from that of control groups. When oocytes were cultured for 1.5 h in medium containing 10% or 30% oviductal fluid before insemination, the incidence of monospermy increased without a decrease in sperm penetration. Preculture of oocytes in medium containing 30% oviductal fluid increased type I cortical granule reaction and increased resistance of the zona pellucida to dissolution by 0.1% (w/v) pronase at the time of sperm penetration. These results suggest that a factor(s) from the oviductal secretion is required for the complete cortical granule reaction and in the modification of the zona pellucida.

Introduction

Recent advances in *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) have increased the availability of mammalian embryos for the study of early zygotic development. However in pigs, the abnormally high incidence of polyspermy after IVM and IVF, was a major problem (Nagai *et al.*, 1984; Mattioli *et al.*, 1988; Funahashi and Day, 1993a). The addition of pig oviductal epithelial cells (Nagai and Moor, 1990) and follicular fluid (Funahashi and Day, 1993b) to the fertilization or pre-fertilization media has been used to reduce the incidence of polyspermy in pig oocytes. The beneficial effects of these fluids in preventing polyspermy may be due to competition between sperm receptors and oviductal and follicular sulfate proteoglycans for zona receptors on boar spermatozoa, which results in a reduction in the number of spermatozoa reaching the surface of the oocytes (Niwa, 1993).

Two major systems during fertilization *in vivo* appear to prevent polyspermy in pigs. In the first system, the female reproductive tract reduces the number of spermatozoa arriving at the surface of the oocytes (Hunter, 1990, 1991). The

second system is called the cortical granule reaction which is the direct preventive response by the oocyte against multiple sperm penetration. Exocytosis of the contents of the cortical granule, which contains hydrolytic enzymes and saccharide components, into the perivitelline space leads to the formation of a cortical granule envelope followed by modification of zona proteins and the inactivation of sperm receptors (Dandekar and Talbot, 1992). Cran and Cheng (1986) have shown that in pigs the number and nature of the release of cortical granules differs *in vitro* from *in vivo* since *in vitro* the dispersal of cortical granule contents into the perivitelline space occurs slowly and incompletely. Buhi *et al.* (1993) detected oviductal secretory proteins in the zona pellucida, perivitelline space and plasma membrane of oviductal pig oocytes and in embryos. It is also known that proteolytic digestion of the zona pellucida is delayed after pig oocytes and embryos have been present in the oviducts (Broermann *et al.*, 1989). Therefore, molecules secreted by the oviduct may be involved during and after the cortical reaction and zona hardening and, thus, in the prevention of polyspermy. The present study was undertaken to determine the effects of oviductal fluid on sperm penetration, cortical granule exocytosis and zona hardening during fertilization *in vitro*.

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Materials and Methods

Preparation of oviductal fluid

Oviductal fluid was collected from sexually mature gilts as described by Archibong *et al.* (1989). Briefly, gilts were anaesthetized on days 17–18 of the oestrous cycle. A cannula from the tygon tubing (i.d.: 1.25 mm; o.d.: 2.25 mm; VWR Scientific, St Louis, MO) 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 every 12 h for 5 days. The fluid from days 20 and 21 of the oestrous cycle was used in this experiment. The fluid was centrifuged at 1000 g for 10 min and the supernatant was filtered and frozen at -20°C until used.

In vitro maturation

Pig oocyte–cumulus complexes with uniform ooplasm and a compact cumulus cell mass were prepared in Hepes-buffered TALP medium containing 0.1% (v/v) polyvinylalcohol (H-TL-PVA, Funahashi *et al.*, 1994). The culture medium for *in vitro* maturation was BSA-free NCSU23 (NCSU23; Petters and Wells, 1993) supplemented with 10% (v/v) pig follicular fluid (pFF, Funahashi and Day, 1993b) 10 iu equine chorionic gonadotrophin ml^{-1} (Intervet America Inc., Millsboro, DE) and 10 iu hCG ml^{-1} (Lypho Med Inc., Rosemont, IL). Fifty oocyte–cumulus complexes were transferred to 500 μl NCSU23, covered with paraffin oil (Fisher Scientific, Pittsburgh, PA) in a four-well culture plate (Nunc, Roskilde), and then cultured for 22 h at 39°C in an atmosphere of 5% CO_2 in air. The oocyte–cumulus complexes were then transferred to 500 μl of NCSU23 without hormonal supplements (Funahashi and Day, 1993a) and cultured for an additional 22 h at 39°C in 5% CO_2 in air.

Fertilization in vitro

A sperm-rich fraction (15 ml) was collected from a boar by the gloved-hand method, and kept at 20°C for 16 h after adding antibiotic–antimycotic solution (Gibco, Grand Island, NY). The semen was washed three times by centrifugation (1000 g for 3 min) with 0.9% (w/v) NaCl supplemented with 1 mg BSA ml^{-1} (Fraction V; Sigma Chemical Co., St Louis, MO). At the end of washing, the pellets containing spermatozoa were resuspended at a concentration of 2×10^8 cells ml^{-1} in modified Medium 199 (Funahashi *et al.*, 1994; mM199, Sigma) at pH 7.8. The sperm suspension was incubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air. Ten oocytes were washed three times with mM199 supplemented with 10 mmol caffeine sodium benzoate l^{-1} and 4 mg BSA ml^{-1} at pH 7.4 and placed into a 50 μl droplet of the mM199 under paraffin oil. Fifty microlitres of diluted preincubated spermatozoa was added to 50 μl of the medium containing the oocytes giving a final sperm concentration of 1×10^6 cells ml^{-1} . Oocytes were co-cultured with spermatozoa for 6 h at

39°C in an atmosphere of 5% CO_2 in air. The oocytes were then transferred to 500 μl of fresh Whitten's medium and cultured at 39°C in an atmosphere of 5% CO_2 in air.

In vivo matured or fertilized eggs

Pro-oestrus gilts were treated with 500 iu hCG on day 19 or day 20 of the oestrous cycle in order to time accurately the onset of ovulation (Hunter, 1972). The gilts were artificially inseminated 39–40 h after the hCG injection. Ovulated oocytes (40–44 h after the hCG injection) and fertilized eggs (8–10 h after artificial insemination) were collected by flushing the oviduct with 30 ml of H-TL-PVA.

Assessment of sperm penetration

At the end of culture, eggs were fixed for 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 $\times 200$ and $\times 400$. 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.

Assessment of cortical granule exocytosis

At specific time points, cumulus cells were removed from the oocytes by repeated pipetting. Denuded eggs were permeabilized in 25% (v/v) glycerol, 50 mmol KCl l^{-1} , 0.5 mmol MgCl_2 l^{-1} , 0.1 mmol EDTA l^{-1} , 1 mmol EGTA l^{-1} , 1 mmol 2-mercaptoethanol l^{-1} , 50 mmol imidazol l^{-1} , pH 6.7, with 4% (v/v) Triton X-100 (Simerly and Schatten, 1993). The eggs were then fixed in methanol at -10°C . After several washes with PBS containing 0.5% (v/v) Triton X-100 and 0.5% (w/v) BSA, eggs were incubated in a blocking solution (0.1 mol glycine l^{-1} , 0.01% Triton X-100 (w/v), 1% (w/v) powdered milk, 0.5% (w/v) BSA and 0.02% (w/v) sodium azide) at 39°C for 1 h. Blocking was followed by incubation in 5 μg fluorescein isothiocyanate (FITC) labelled lectins ml^{-1} (*Lens culinaris* agglutinin; Sigma) at 39°C for 1 h. DNA was detected fluorescently by exposure to 20 μg propidium iodide ml^{-1} (Sigma) for 1 h. The eggs were then mounted under a coverslip with antifade mounting medium (Vectashild, Vector Lab., Burlingame, CA) to retard photobleaching.

Slides were examined using laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 600 equipped with a Krypton–argon ion laser for the simultaneous excitation of fluorescein for cortical granules and propidium iodide for DNA. The images were recorded digitally and archived on an erasable magnetic optical disk.

Assessment of solubility of zonae pellucidae

At specific time points the oocytes were washed in H-TL-PVA. Ten microlitres of H-TL-PVA containing 8–10 eggs were transferred to 500 μl of 0.1% (w/v) pronase solution in

Table 1. Sperm penetration and cortical granule reaction in pig oocytes matured *in vitro* or *in vivo*

Maturation/ fertilization	Number of oocytes (%)		Mean number of sperm penetrating	Cortical granule exocytosis (%)		
	Examined	Penetrated		Type I	Type II	Type III
<i>In vivo/in vivo</i>	11	10 (91)	1.0 ± 0.0	10 (100)	—	—
<i>In vivo/in vitro</i> *	27	23 (85)	1.6 ± 0.3 ^a	18 (72) ^a	3 (13)	2 (9) ^a
<i>In vitro/in vitro</i> *	43	41 (95)	2.6 ± 0.5 ^b	10 (24) ^b	5 (12)	26 (63) ^b

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

*The oocytes matured *in vivo* or *in vitro* were inseminated with the same semen at the same time.

Dulbecco's phosphate buffered saline (dPBS). Zonae pellucidae were continually observed for dissolution at room temperature with an inverted microscope. The time between placement of the eggs in dPBS containing pronase and when the zona pellucidae of half of the eggs were no longer visible at $\times 200$ magnification was designated as the zona pellucida dissolution time for those groups.

Experimental design

Three experiments were designed as follows:

Experiment 1. Sperm penetration and cortical granule reaction were compared following *in vitro* fertilization of *in vivo* and *in vitro* matured oocytes. Six hours after insemination, eggs were fixed to determine distribution of cortical granules and sperm penetration.

Experiment 2. The effect of various concentrations of oviductal fluid in the fertilization media on sperm penetration and cortical granule reaction was examined. At the end of the maturation culture, oocytes were inseminated in fertilization media containing 0, 0.1, 1 and 10% oviductal fluid. A rapid decrease in sperm motility had been observed in the medium containing 10% oviductal fluid, and therefore 10% added oviductal fluid was selected to be the maximum concentration and 0.1% as a minimum concentration. Six hours after insemination, the distribution of the cortical granules was determined. Sperm penetration and pronuclear formation were determined 12 h after insemination.

Experiment 3. At the end of maturation culture, oocytes were cultured in the fertilization medium supplemented with 0, 10, 30% oviductal fluid, or in 100% oviductal fluid for 1.5 h before insemination. Three or six hours after insemination in medium without added oviductal fluid, the distribution of cortical granules and the zona pellucida dissolution time were determined. Sperm penetration and pronuclear formation were determined in a second sample of oocytes 12 h after insemination. High concentrations of oviductal fluid were examined since *in vivo* the oocytes are bathed in oviductal fluid before fertilization.

Statistical analyses

Statistical analyses of data from three replicate trials were carried out by 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 penetrating the zona was expressed as mean \pm SEM. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Experiment 1: cortical granule reaction in oocytes matured *in vivo* or *in vitro*

There was no difference ($P > 0.05$) in the incidence of sperm penetration between oocytes matured *in vivo* and *in vitro* (Table 1). The mean number of penetrating sperm was significantly higher ($P < 0.05$) in oocytes matured *in vitro* than in those matured *in vivo* (Table 1). After labelling with FITC-labelled lectin, fluorescence spots were distributed in the entire cortical cytoplasm of mature unfertilized oocytes (Fig. 1a–c). The distribution of cortical granule exudate following sperm penetration (6 h after insemination) could be classified into three groups: type I when a complete cortical granule reaction was observed and the cortical granule exudate was evenly distributed in the entire perivitelline space (Fig. 1d); type II when a complete cortical granule reaction was observed and cortical granule exocytosis partially filled the perivitelline space (Fig. 1e) and type III when the cortical granule reaction was incomplete and the cortical granule exudate was unevenly distributed into the perivitelline space (Fig. 1f). All oocytes fertilized *in vivo* showed type I cortical granule exocytosis (Table 1). The incidence of oocytes with type I exocytosis following IVF was higher in the oocytes matured *in vivo* than those matured *in vitro* (Table 1).

Experiment 2: effect of oviductal fluid in the fertilization medium

Supplementation of oviductal fluid at concentrations of 1% and 10% to the fertilization medium decreased sperm penetration and increased the incidence of monospermy (Table 2). The distribution of cortical granule exocytosis after sperm penetration did not differ between control and treatment (1% oviductal fluid) groups (Fig. 2). Exocytosis of cortical granules was not examined in the group with 10% oviductal fluid added to the fertilization medium due to the low rate of sperm penetration (9%).

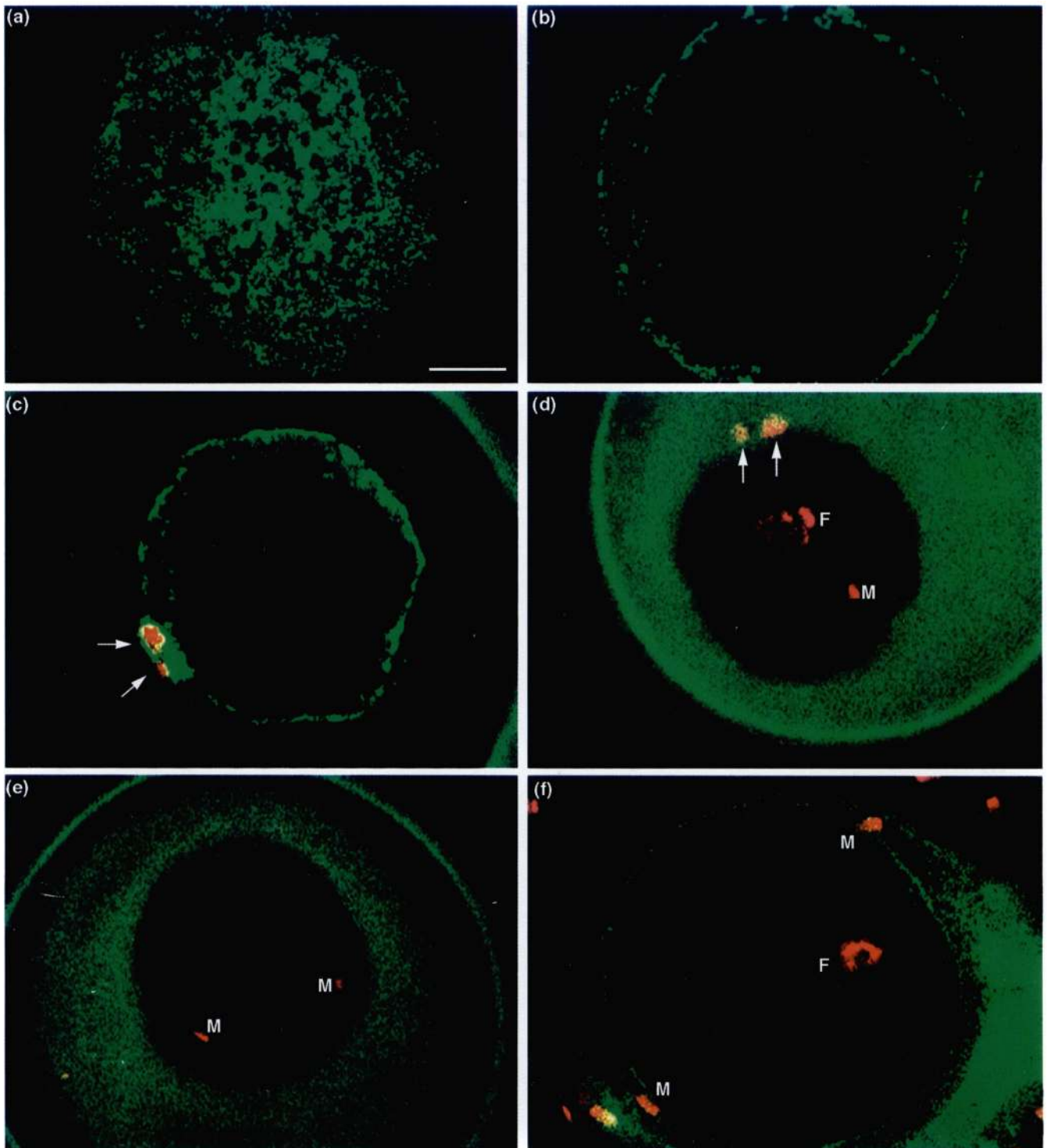


Fig. 1. Confocal images of cortical granules in pig oocytes matured *in vitro* before and after sperm penetration. Green and red images show cortical granules and DNA, respectively. (a, b) The same zona free-oocyte at different focal points. (a) The depth of field in this optical section is about 10–15 μm . The surface of the matured oocyte was covered by numerous fluorescence spots. (b) The depth of field in this optical section is about 40–50 μm . Focused at the centre of the cell, cortical granules were observed in the plasma membrane. (c) A zona-intact unfertilized oocyte. Fluorescence was not detected in the perivitelline space before sperm penetration. Thick cortical granule layers were observed around metaphase II chromosomes and the polar body (arrows). (d) Type I distribution of cortical granule exudate of an oocyte matured *in vitro* 6 h after insemination. The exudate is distributed evenly in the entire perivitelline space. Polar bodies (arrows), female pronucleus (F) and sperm head (M) are seen. (e) Type II distribution of cortical granule exudate of an oocyte matured *in vitro* 6 h after insemination. A complete cortical reaction has occurred, but the exudate is not distributed in the entire cytoplasm. (f) Type III cortical reaction, that is, an incomplete reaction was observed. Scale bar represents 25 μm .

Table 2. Sperm penetration of pig oocytes *in vitro* in the presence of oviductal fluid at different concentrations in the fertilization media

Concentration of oviductal fluid	Number of oocytes		Number of monospermic oocytes (%)	Mean number of spermatozoa in penetrated oocytes	Number of oocytes that formed a male pronucleus (%)
	Examined	Penetrated (%)			
0.0%	156	142 (91) ^a	17 (12) ^a	4.1 ± 0.7	96 (68)
0.1%	94	78 (82) ^{a,b}	11 (14) ^a	3.4 ± 0.9	51 (65)
1.0%	173	127 (72) ^b	39 (31) ^b	2.2 ± 0.5	93 (73)
10.0%	89	7 (9) ^c	7 (100) ^c	1.0 ± 0.0	3 (43)

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

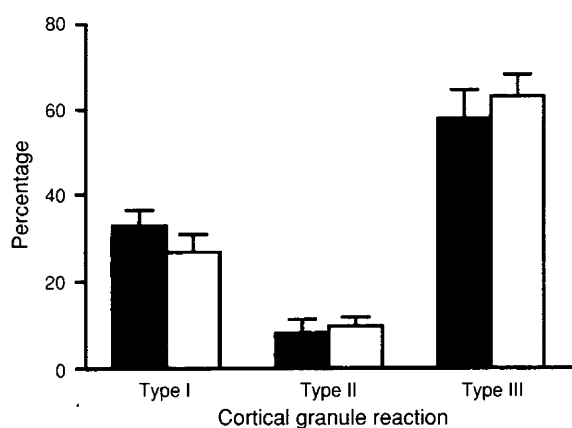


Fig. 2. Effect of pig oviductal fluid on the cortical granule reaction 6 h after insemination. Pig oocytes were fertilized in the (■) absence ($n = 59$) or (□) presence ($n = 62$) of 1% oviductal fluid. Type I reaction: cortical granule reaction complete, exudate evenly distributed into the perivitelline space; type II: reaction complete but exudate only partially distributed; type III: reaction incomplete.

Experiment 3: effects of oviductal fluid in preculture medium

Preculture of pig oocytes in medium containing 10% or 30% oviductal fluid significantly increased the percentage of monospermic penetration without decreasing sperm penetration (Table 3). The mean number of spermatozoa penetrating was also lower in oocytes precultured in 100% oviductal fluid than in the control group. The incidence of oocytes with type I exocytosis increased ($P < 0.05$) when oocytes were cultured for 1.5 h in medium containing 30% oviductal fluid before insemination (Fig. 3). Exocytosis was examined only in the 30% treatment group since the frequency of monospermy did not differ among the three treatment groups supplemented with oviductal fluid. When oocytes were exposed to medium containing 30% oviductal fluid for 1.5 h, the zona pellucida dissolution time increased ($P < 0.05$) from 1.2 ± 0.3 to 6.4 ± 0.8 min. The zona pellucida dissolution time increased in oocytes precultured before sperm penetration, but was unchanged in control groups (Fig. 4).

Discussion

During sperm penetration, cortical granules fuse with the overlying oolemma and release their contents into the perivitelline space. In pigs, the cortical granule exudate appears to block polyspermy by changing the properties of the zona pellucida. In the present study, incomplete cortical granule exocytosis in pig oocytes, matured and fertilized *in vitro* has been demonstrated. This observation is consistent with the study by Cran and Cheng (1986) using electron microscopy, who demonstrated that the cortical granule reaction was slower and incomplete in pig oocytes fertilized *in vitro* compared with those fertilized *in vivo*. Incomplete dispersal of exudate upon exocytosis has been reported in cattle during IVF (Hyttel *et al.*, 1988, 1989). Dandekar and Talbot (1992) also reported that polyspermic human oocytes fertilized *in vitro* contain aggregated cortical granules rather than dispersed cortical granule exudate, whereas monospermic hamster and mouse oocytes fertilized *in vivo* possess well formed cortical granule material in the perivitelline space.

It has been suggested that in pig ova the cortical granule reaction resulting from current IVM and IVF techniques is not effective in preventing polyspermy (Sirard *et al.*, 1993). Although electrical stimulation induces the cortical reaction in pig ova matured *in vitro* (Sun *et al.*, 1992), it does not block sperm penetration (Funahashi *et al.*, 1993). As shown in the study reported here, the failure to distribute the cortical granule exudate after sperm penetration *in vitro* can lead to the defective formation of a cortical granule envelope, and thus to an ineffective block to polyspermy. Alternatively, the zona pellucida may be less responsive to the inadequate cortical granule distribution following exocytosis. It was demonstrated that IVF after IVM in a low concentration of NaCl increased the width of the perivitelline space and reduced the incidence of polyspermic penetration (Funahashi *et al.*, 1994). A high concentration of NaCl in the maturation medium has detrimental effects on microfilament organization (H. Funahashi *et al.*, 1996). Microfilaments are one of the major cytoskeletons in mammalian oocytes and represent about 2% of total protein in the oocyte (Longo, 1987). In sheep, Le Guen *et al.* (1989) have shown that treatment of matured oocytes with cytochalasin D causes profound internal structural changes to the cytoplasm of the cortical granule and, in some regions, induces a loss of association with the plasma membrane. Abnormal patterns of

Table 3. Effect of oviductal fluid in preculture medium on sperm penetration of pig oocytes

Concentration of oviductal fluid (%)	Number of oocytes			Mean number of sperm penetrating	Number of oocytes that formed a male pronucleus (%)
	Examined	Penetrated	Monospermic		
0	81	71 (88)	9 (13) ^a	3.9 ± 0.7 ^a	51 (71)
10	87	71 (81)	32 (45) ^b	2.2 ± 0.5 ^b	46 (64)
30	76	63 (77)	29 (49) ^b	1.5 ± 0.3 ^c	36 (61)
100	56	52 (87)	16 (33) ^b	1.9 ± 0.4 ^{b,c}	29 (60)

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

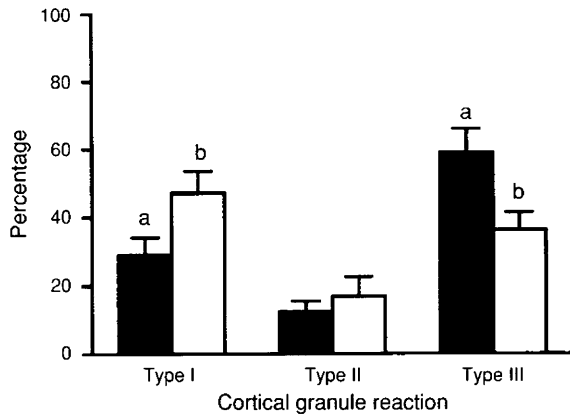


Fig. 3. Effect of preculture of pig oocytes in the (■) absence ($n = 49$) or (□) presence ($n = 57$) of 30% oviductal fluid before insemination on the cortical granule reaction 6 h after insemination. Type I reaction: cortical granule reaction complete, exudate evenly distributed into the perivitelline space; type II: reaction complete but exudate only partially distributed; type III: reaction incomplete. Bars with different superscripts are significantly different ($P < 0.05$).

microfilament organization were demonstrated in pig oocytes after IVM compared with those matured *in vivo* (Kim *et al.*, 1996a). The abnormalities of microfilament organization seem to be closely related to the culture system during *in vitro* maturation (H. Funahashi *et al.*, 1996) and oocyte ageing (Kim *et al.*, 1996). Collectively, inadequate culture conditions during IVM may disturb the function of microfilaments, and thus cause an incomplete cortical reaction following sperm penetration. A study on the relationship between microfilaments and cortical granule movement and exocytosis during maturation, artificial activation and fertilization is in progress in our laboratory.

In the present study, epifluorescence microscopy using lectin was used to observe the distribution of cortical granules during sperm penetration. Fluorescence-labelled lectin has been used to localize specific sugars in mammalian cortical granules (Cherr *et al.*, 1988; Ducibella *et al.*, 1990; Long *et al.*, 1994). This method has great advantages over observation of cortical granules by electron microscopy, since it allows rapid visualization and examination of cortical granule distribution in oocytes during fertilization. Improved techniques in terms of resolution and the ability to section the egg optically have been assessed using laser-scanning confocal microscopy (Yoshida *et al.*, 1993; Hoodbhoy and Talbot, 1994).

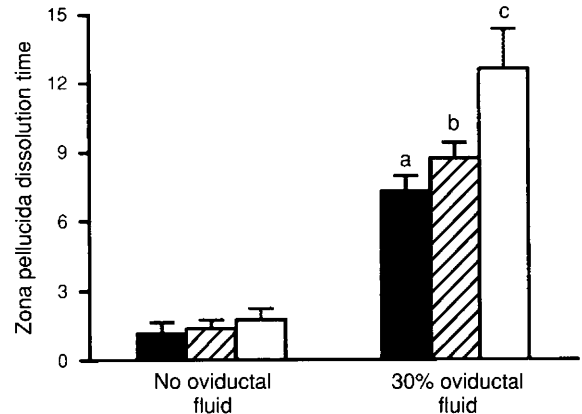


Fig. 4. Effect of preculture of pig oocytes in the absence or presence of 30% oviductal fluid before insemination on zona solubility (■) 0, (▨) 3 and (□) 6 h after insemination. Bars with different superscripts are significantly different ($P < 0.05$).

The present study showed that the presence of oviductal fluid in the fertilization medium reduced sperm penetration and increased the percentage of monospermy in a dose-dependent manner. Nagai and Moor (1990) suggested that macromolecules secreted from pig oviductal cells reduced polyspermy in oocytes fertilized *in vitro* by means of an interaction with the fertilizing spermatozoa. The results reported here also indicate that oviductal fluid may affect the sperm cell via a reduction in sperm penetration rather than oocyte condition since the type of cortical reaction was unchanged during sperm penetration. Mo *et al.* (1991) detected a zona-binding protein of low molecular mass, which is probably derived from seminal fluid, in the acrosomal cap region of the sperm head. Binding of the zona-binding glycoproteins of boar spermatozoa with zona pellucida glycoproteins is inhibited by sulfated polymers, such as dextrane sulfate, polyvinylsulfate and heparin (Parry *et al.*, 1992; Jones, 1991). Therefore glycoproteins (probably in the sulfated form) in oviductal fluid may reduce the attachment of spermatozoa to the zona pellucida, resulting in a low sperm penetration rate.

In the study presented here, the preculture of oocytes in the presence of 10% and 30% oviductal fluid increased the incidence of monospermy without decreasing sperm penetration. There are two possible explanations for the effect of oviductal fluid on the oocyte in preventing polyspermy *in vivo* (Hunter, 1991, 1994). Glycoproteins secreted from the pig oviduct bind

to the zona pellucida of newly ovulated pig oocytes (Brown and Cheng, 1986) and then these glycoproteins act to reduce the rate of simultaneous penetration of the zona pellucida. An alternative interpretation is that a glycoprotein deposition on the zona pellucida, followed by its entry into the perivitelline space, facilitates either a more synchronous exocytosis of cortical granule contents or expedites and augments physiological responses of the zona substance to cortical granule material. The study presented here supports the second possibility since preculture of oocytes in medium containing oviductal fluid increased type I cortical granule exocytosis, and increased the resistance of the zona pellucida to digestion by 0.1% pronase.

In summary, the present study demonstrated incomplete cortical granule exocytosis in oocytes matured and fertilized *in vitro*. The addition of oviductal fluid to the fertilization medium decreased sperm penetration without changing the distribution of cortical granule exudate in the perivitelline space during sperm penetration. Preculture of oocytes with a high concentration (30%) of oviductal fluid increased complete cortical granule exocytosis and induced zona hardening, suggesting a role for oviductal fluid in the cortical reaction and zona hardening.

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