Study of Zona Pellucida Function During *In Vitro* Fertilization in Pigs

ブタ体外受精における透明帯の機能に関する研究

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Contents

ABSTRACT

Efficient generation of viable porcine embryos will contribute to research in reproductive physiology, agriculture and biotechnology, including cloning and transgenesis in pigs, and the establishment of pigs as laboratory animals for human disease models. However, polyspermy still occurs with high frequency during *in vitro* fertilization (IVF) in pigs and polyspermy is considered to be a very troublesome obstacle to efficient production of normal porcine embryos. The zona pellucida (ZP) is considered to be important for prevention of polyspermy in mammalian oocytes. After sperm penetratration into ooplasm, contents of the cortical granules were released to the perivitelline space (PVS), they act on the ZP, and cause biochemical and structural changes of ZP that make ZP lost sperm ability to bind ZP and penetration ability of sperm previously bound to the ZP (zona reaction). However, the function(s) with regard to sperm penetration or prevention of polyspermy is not well understood in pigs.

In the present study, the first series of experiments was conducted to investigate the effects of the ZP on sperm penetration during IVF. I collected *in vitro* matured oocytes with a first polar body (ZP+ oocytes). Some of them were freed from the ZP (ZP− oocytes) by two treatments (pronase and mechanical pipetting), and the effects of

these treatments on sperm penetration parameters (sperm penetration rate and numbers of penetrated sperm per oocyte) were evaluated. There was no significant difference in the parameters between the two groups. Secondly, I compared the sperm penetration parameters of ZP+ and ZP− oocytes using frozen-thawed epididymal spermatozoa from four boars. Sperm penetration into ZP+ oocytes was found to be accelerated relative to ZP− oocytes. Thirdly, I evaluated the sperm penetration of ZP+ and ZP− oocytes at 1−10 h after IVF (co-incubation of gametes for 3 h). The proportions of oocytes penetrated by sperm increased significantly with time in both groups; however, the number of penetrated sperm per oocyte did not increase in ZP− oocytes. Finally, I performed IVF using ZP− oocytes divided into control (3 h) and prolonged gamete co-incubation (5 h) groups. Significantly greater numbers of sperm penetrated in the 5 h group than in the control group. These results suggest that the ZP and oolemma are not competent factors for prevention of polyspermy in the porcine IVF system using in our laboratory. Furthermore, the presence of the ZP accelerates sperm penetration into the ooplasm.

The second series of experiments was conducted to evaluate the detail functions of the ZP for sperm penetration. Firstly, I investigated the effects of the ZP on sperm binding and acrosomal status. I evaluated the numbers of sperm bound to the ZP in

ZP+ oocytes and oolemma in ZP− oocytes. Acrosomal statuses of these binding sperm were also evaluated. Furthermore, I evaluated the numbers and acrosomal statuses of sperm presenting in the ZP and perivitelline space (PVS) using ZP+ and ZP− oocytes. More sperm bound to the ZP than to the oolemma. The average number of sperm present in the PVS was 0.44−0.51 per oocyte, and all sperm had lost their acrosomes. I found that the sperm in the PVS, in other words, the sperm passing through the ZP can fuse with oolemma with high efficiency. It may be considered that the sperm are induced some kind of factors involved in sperm-oolemma fusion by passing through the ZP. So in the second experiment, I focused on IZUMO, a critical factor involved in sperm-oolemma fusion, and investigated the effects of the ZP on immunological detection of IZUMO. The proportion of sperm that were immunopositive for anti-IZUMO antibody was significantly higher after they were passing or had passed through the ZP. Furthermore, I performed IVF in the medium supplemented anti-IZUMO antibody to investigate the importance of IZUMO to sperm penetration in pigs. Addition of anti-IZUMO antibody to the fertilization medium significantly inhibited the penetration of sperm into ZP− oocytes. Finally, I investigated whether the ZP induces the synthesis of IZUMO in sperm using two kinds of protein synthesis inhibitors, chloramphenicol (CP) and cycloheximide (CH). It has been reported that

eukaryotic cells including spermatozoa have two kinds of ribosomes, mitochondrial 55S and cytoplasmic 80S ribosomes. I hypothesized that CP and CH inhibits 55S and 80S ribosomes, respectively, resulting in inhibition of mRNA translation in sperm. Addition of CP and CH to fertilization medium had no effect on immunological detection of IZUMO during IVF. These results indicate that the ZP induces the acrosome reaction, which is associated with the functional exposure of IZUMO, resulting in completion of fertilization in pigs. It is suggested that IZUMO may not be synthesized during IVF and undergoes some modifications resulting in exposure of IZUMO during passing through the ZP.

In the present study, I elucidated the ZP, accelerates functional exposure of IZUMO resulting successful fertilization. The ZP supports success of normal fertilization not only by being barrier to extra sperm penetration, but also by preparing the condition of sperm for fusing with oolemma. Research of mechanisms controlling fertilization in pigs, such as preventing polyspermy and sperm-oolemma fusion, are expected to contribute improving IVP system in pigs and also to the research of biotechnology in other species.

GENERAL INTRODUCTION

Efficient generation of viable porcine embryos will contribute to research in reproductive physiology, agriculture, and biotechnology, including cloning and transgenesis in pigs. Recently, the value of pigs as laboratory animals has become widely recognized, and porcine embryonic stem cells would be helpful for the establishment of human disease models. The application of efficient production of normal embryos is also expected for improving studies of porcine embryonic stem cells.

In vitro production (IVP) system for porcine embryos has been dramatically developed (Abeydeera 2002, Wheeler *et al*. 2004, Kikuchi 2004, Kikuchi *et al*. 2008). IVP system, including *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and their subsequent *in vitro* culture (IVC), is fundamental procedure for the production of embryos *in vitro*. Motlik and Fluka (1974) investigated the ability of IVM oocytes to be fertilized and reported that IVM oocytes were able to be fertilized *in vivo*. Sperm are incapable of fertilizing oocytes immediately after ejaculation (Austin 1951). Sperm needs to acquire capability to fertilize, which is termed "capacitation" (Chang 1951). In *in vivo*, sperm are capacitated in female genital tracts

(Hunter *et al*. 1968, 1972, 1973, Hancock *et al*. 1968). Based on this finding, Iritani *et al*. (1978) reported successful IVF using epididymal and ejaculated spermatozoa preincubated in isolated female genital tracts. Thereafter, Nagai *et al*. (1984) reported sperm penetration using epididymal and ejaculated spermatozoa preincubated in a chemically defined medium, which is the first paper successful fertilization in the complete *in vitro* condition. Recent study suggests intracellular Ca^{2+} and HCO_3^- regulate sperm capacitation (Breitbart 2002). In earlier studies, freshly ejaculated semen was the main source of sperm for IVF because of the difficulty in cryopreserving sperm in pigs. In recent years, cryobiological studies have led to the improvement of cryopreservation protocols (Clarke and Johnson 1987, Nagai *et al*. 1988, Kikuchi *et al*. 1998, Gil *et al*. 2008, Okazaki *et al*. 2012). These advances have allowed most IVF laboratories to use frozen-thawed spermatozoa in order to standardize the male factor and minimize the variability among trials in IVF experiments. Furthermore, the modification of the IVF conditions has yielded high penetration and blastocyst development rates (modifying the co-incubation time). (Grupen and Nottle 2000, Funahashi and Romar 2004, Gil *et al*. 2004)

The developmental competence and viability of IVM-IVF oocytes after IVC have been confirmed (Mattioli *et al*. 1989, Yoshida *et al*. 1990) and the birth of piglets has

been accomplished from IVM-IVF embryos after IVC to the two- to four-cell stages (Yoshida *et al*. 1993) or to the eight-cell to morula stage (Abeydeera *et al*. 1998). However, in those days, the quality of IVP blastocysts was low (Kikuchi *et al*. 1990). Many laboratories had been trying to overcome incompleteness of the IVP system by aiming for successful pregnancies to term after the transfer of blastocysts to recipients, however almost all of these challenges had resulted in failure. Kikuchi *et al*. (2002) modified IVP system and they succeeded in production of piglets after transfer of blastocysts produced *in vitro* to recipients and established procedure for the production of high-quality porcine blastocysts.

Although porcine IVP has been established in the point of viable blastocyst production, polyspermy still occurs with high frequency (Funahashi 2003, Gil *et al.* 2010). Polyspermy is considered to be a very troublesome obstacle to the efficient production of normal porcine embryos because although polyspermic oocytes can develop to blastocysts, their ploidy becomes abnormal (Han *et al*. 1999, Somfai *et al*. 2008). Up to now, some studies have focused on reducing polyspermy. It has been reported that exposure of gametes to oviductal epithelial cells and/or oviductal secretions can reduce polyspermy (Nagai and Moor *et al*. 1990, Kim *et al*. 1996, Wang *et al*. 2003, Coy *et al*. 2008). Kim *et al.* (1996) reported that addition of 1.0% oviductal

fluid to the fertilization medium increased monospermy. Coy *et al.* (2008) reported that exposure of oocytes to undiluted oviductal fluid (1 oocyte per microliter of fluid) for 30 min before performing IVF decreased polyspermy significantly. Furthermore, Nagai and Moor (1990) demonstrated that 2.5 h co-culture of sperm and oviduct cells reduces polyspermy. Other studies also have attempted to modify the equipment used for IVF to regulate the number of penetrable sperm near the oocytes, resulting in reduction of polyspermy, for example, the climbing over a wall (COW) method (Funahashi and Nagai 2000), biomimetic microchannel IVF system (microfluidic culture system) (Wheeler *et al*. 2004), straw IVF (Li *et al*. 2003). These methods has been proposed as ways to separate sperm and mature oocytes, and ensure that only motile sperm gain access to the oocytes, mimicking the physical conditions of fertilization *in vivo*. However, the mechanism responsible for polyspermy is still not well understood, and efforts to clarify it have been limited.

The zona pellucida (ZP) is considered to be important for prevention of polyspermy in mammalian oocytes. The most accepted mechanism for prevention of polyspermy is modification of the ZP through release of contents from cortical granules (CG) (Sun 2003, Wang *et al*. 2003). Sperm penetration induces the release of intracellular Ca^{2+} and that induces CG exocytosis to the perivitelline space (PVS)

(cortical reaction). After CG exocytosis, the CG contents act on the ZP directly; causing biochemical and structural changes that make ZP lost sperm ability to bind ZP and penetration ability of sperm previously bound to the ZP (zona reaction). To establish an efficient method(s) for producing normal porcine embryos by reduction of polyspermy, it has become necessary to clarify precisely the role played by the ZP in normal fertilization.

Some mechanisms of sperm-egg fusion and their corresponding factors have been reported. Until today, some fusion-related proteins on mammalian sperm have been discussed. Fertilin was reported as PH-30 which is a sperm surface protein involved in sperm-egg fusion (Blobel *et al*. 1992). Fertilin consists of two subunits, fertilin-α (ADAM1b) and fertilin-β (ADAM2). Sperm from ADAM2 knockout mouse are defective in migrating into the oviduct and binding to the ZP, however they are able to fuse with oolemma (Cho *et al.* 1998). Fertilin is not critical factor for sperm-egg fusion. Cyritestin (ADAM3) (Nishimura *et al*. 2007) and CRISP (cysteine-rich secretory protein) (Cohen *et al*. 2008) were also hoped for the sperm-egg fusion factors. However, sperm from ADAM3 knockout mouse had fusion ability with oolemma (Shamsadin *et al*. 1999, Nishimura *et al*. 2001). They were defective in migrating into the oviduct and binding to ZP, similar to sperm from ADAM2 knockout mouse

(Yamaguchi *et al*. 2009). CRISP1, a member of the CRISP family, was not critical sperm-egg fusion factor, neither. IVF assays showed that sperm from CRISP1 knockout mouse exhibited a significantly reduced ability to penetrate both ZP-intact and ZP-free oocytes, however some of them were able to fuse with oolemma. Furthermore, CRISP1 knockout male mice were fertile, and they presented no significant differences in the average litter size compared with control mice (Da Ros *et al*. 2008). Equatorin, which is the antigenic molecule of the monoclonal antibody mMN9, is also one of the sperm-egg fusion related factors of sperm. Addition of mMN9 to fertilization medium reduced sperm penetration in mice (Toshimori *et al*. 1998). Equatorin is localized at the equatorial segment after acrosome reaction (AR). Analysis of knockout mice is being required. Recently, Izumo (described as “IZUMO” in other species except for mice), one of the membrane proteins on sperm, was discovered as critical factor for sperm-egg fusion in mice and human (Inoue *et al*. 2005). In mice, characteristics of Izumo, such as localization (Yamashita *et al*. 2007) and isoforms (Ellerman *et al*. 2009), are well studied. Ellerman *et al.* (2009) identified three other isoforms (Izumo2−4) whose N-terminal domains showed significant homology to that of the original Izumo (Izumo1), and suggested that these isoforms form protein complexes such as homodimers. However, until today, there is only one

research concerning IZUMO in pigs (Kim *et al*. 2012). More studies of porcine IZUMO are needed.

Research of mechanisms controlling fertilization in porcine species, such as preventing polyspermy and sperm-oolemma fusion, are expected to contribute improving IVP system in pigs and research of biotechnology in other species. Because of limitation in the ethical issues, it is difficult to use human oocytes for research in fertilization mechanisms in human species and human regenerative medicine. Researches in porcine species as an experimental model are also expected to be applied to research in human species, resulting in improvement of medical and pharmaceutical industry including human.

I hypothesized that the ZP has important role(s) to control sperm delivery to oolemma. Furthermore, the research in factors involved in sperm-oolemma fusion is necessary for the further control of fertilization. Therefore, in ARTICLE 1, I evaluated the roles of the ZP and oolemma during IVF to clarify the mechanism(s) of polyspermy in pigs. In ARTICLE 2, I investigated the ZP function for sperm binding, acrosome status, and mechanism of IZUMO on sperm penetration.

ARTICLE 1

Evaluation of Zona Pellucida Function for Sperm Penetration

ABSTRACT

In porcine oocytes, the function of the zona pellucida (ZP) with regard to sperm penetration or prevention of polyspermy is not well understood. In the present study, I investigated the effects of the ZP on sperm penetration during *in vitro* fertilization (IVF). I collected *in vitro* matured oocytes with a first polar body (ZP+ oocytes). Some of them were freed from the ZP (ZP− oocytes) by one of two treatments (pronase and mechanical pipetting), and the effects of these treatments on sperm penetration parameters (sperm penetration rate and numbers of penetrated sperm per oocyte) were evaluated. There was no significant difference in the parameters between the two groups. Secondly, I compared the sperm penetration parameters of ZP+ and ZP− oocytes using frozen-thawed epididymal spermatozoa from four boars. Sperm penetration into ZP+ oocytes was found to be accelerated relative to ZP− oocytes. Thirdly, I evaluated the sperm penetration of ZP+ and ZP− oocytes at 1−10 h after IVF (3 h gamete co-incubation). The proportions of oocytes penetrated by sperm increased significantly with time in both groups; however, the number of penetrated sperm per oocyte did not increase in ZP− oocytes. Finally, I performed IVF using ZP− oocytes divided into control (3 h) and prolonged gamete co-incubation (5 h) groups. Greater

numbers of sperm penetrated in the 5 h group than in the control group. These results suggest that the ZP and oolemma are not competent factors for prevention of polyspermy and also that the presence of the ZP accelerates sperm penetration into the ooplasm in the present porcine IVF system using in our laboratory.

INTRODUCTION

In human oocytes, malfunction of the ZP (Paz *et al*. 2008) and anti-zonal antibodies (Szczepanska *et al*. 2001, Ulcova-Gallova *et al*. 2004, Al-Daghistani and Fram 2009) have been reported to be a cause of infertility and failure of IVF, and abnormality of the ZP is also one of the causes of polyspermic penetration (Wang *et al*. 2003). It is expected that spermatozoa can easily penetrate into an oocyte after removal of the ZP. In mice, Wolf *et al*. (1976) reported that the rate of oocytes penetrated by sperm in zona-free oocytes prepared by mechanical techniques was higher than that of zona-intact oocytes. Contrary to this observation, the ZP protects oocytes and embryos mechanically during fertilization and development. Therefore, it is suspected that removal of the ZP has detrimental effects on normal fertilization and development of embryos before implantation. However, because healthy offspring have been born to humans and pigs after transfer of blastocysts that have developed *in vitro* from ZP-free oocytes (Wu *et al*. 2004, Shu *et al*. 2010), it is hypothesized that removal of the ZP is an efficient method for overcoming infertility caused by ZP abnormality in humans and other mammals. On the other hand, the ZP has been shown to play an important role in the successful fertilization of mammalian oocytes, for example, in induction of the

acrosome reaction (Berger *et al*. 1989, Wu *et al*. 2004), efficient effects on sperm binding (Fazeli *et al*. 1997), and prevention of polyspermy (Bleil and Wassarman 1980, Wang *et al*. 2003, Canovas *et al*. 2009). Removal of the ZP may have unexpected influences on these functions.

In porcine oocytes, polyspermy occurs with high frequency and is considered to be an obstacle for efficient IVP of normal embryos (Funahashi 2003, Kikuchi *et al*. 2009). In mammalian oocytes, the most accepted mechanism for prevention of polyspermy is modification of the ZP through release of cortical granules (zona reaction) (Sun 2003, Wang *et al*. 2003). After these biochemical and structural changes, the ZP loses its ability to bind and be penetrated by sperm (Miller *et al*. 1993, Aviles *et al*. 1997, Burkart *et al*. 2012). It is also known that the porcine ZP does not prevent polyspermy, especially in *in vitro* matured porcine oocytes (Funahashi 2003); however, the function of the ZP in this species remains insufficiently understood.

In ARTICLE 1, I examined the roles of the porcine ZP in sperm penetration and polyspermy prevention. Firstly, I evaluated the effects of pronase treatment of the ZP on sperm penetration. Pronase is a protease purified from the extracellular fluid of *Streptomyces griseus* (Nomoto and Narahashi 1959) that has been used widely to dissolve/remove the ZP in mammals. Secondly, I investigated the function of the ZP in

sperm penetration using porcine oocytes from which the ZP had been removed. Thirdly, to elucidate whether the ZP and/or oolemma functions to prevent polyspermy, I evaluated the penetration parameters of oocytes with or without the ZP. Finally, I focused on the function of the oolemma in prevention of polyspermy.

MATERIALS AND METHODS

Oocyte collection and IVM

Collection and IVM of porcine oocytes were carried out as reported previously (Kikuchi *et al*. 2002). In brief, porcine ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COCs) were collected from follicles 2–6 mm in diameter in Medium 199 (M199; with Hank's salts, Sigma-Aldrich Corp., St Louis, MO, USA) supplemented with 5% (v/v) fetal bovine serum (Gibco, Life Technologies Corp., Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium (Sigma-Aldrich), and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich). About 40 COCs were cultured in 500 μl of maturation medium for 20–22 h in four-well dishes (Nunclon Multidishes; Thermo Fisher Scientific, Waltham, NA, USA). The medium employed was modified North Carolina State University (NCSU)-37 solution (Petters and Wells 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 mM β-mercaptoethanol, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP; Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical Co., Ltd., Tokyo,

Japan), and 10 IU/ml hCG (Puberogen 1500 U; Novartis Animal Health, Tokyo, Japan). The COCs were subsequently cultured for 24 h in maturation medium without dbcAMP and hormones. Maturation culture was carried out at 39°C under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5% , and 90% respectively (5% CO_2 and 5% O_2). After culture, cumulus cells were removed from the oocytes by treatment with 150 IU/ml hyaluronidase (Sigma-Aldrich) in M199 and gentle pipetting. Denuded oocytes with the first polar body were harvested under a stereomicroscope and used as *in vitro* matured and ZP-intact oocytes (ZP+ oocytes).

Preparation of the ZP-free oocytes

I obtained ZP-free oocytes by the following two methods. 1) Matured oocytes were exposed to 0.5% (w/v) pronase (Sigma-Aldrich, P-8811) in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for 20−30 sec (Peura and Vajta 2003). Oocytes with an expanded and deformed ZP were then transferred to M199 without pronase and freed completely from the ZP by gentle pipetting. After 1 h of incubation in IVM medium at 39°C under 5% CO_2 and 5% O_2, these ZP-free oocytes, termed "pZP− 1 h oocytes," were used for further experiments. 2) The ZP was removed mechanically using a micromanipulator (MMO-204, Narishige,

Tokyo, Japan) without pronase treatment, employing a modification of a method designed for mouse oocytes (Yamagata *et al*. 2002). First, I stabbed the ZP with a glass needle and formed a slit in it. Next, I aspirated the cytoplasm into a holding pipette. These ZP-free oocytes were termed "mZP− oocytes."

IVF and evaluation of fertilization

The oocytes in all groups were subjected to IVF, as described previously (Kikuchi 2002). In brief, epididymides were isolated from Landrace boars, and epididymal spermatozoa were collected from them and frozen (Kikuchi *et al*. 1998). Spermatozoa were thawed and preincubated for 15 min in Medium 199 with Earl's salts (Gibco) adjusted to pH 7.8 (Nagai *et al*. 1988). Oocytes were transferred to fertilization medium for porcine oocytes (Pig-FM) (Suzuki *et al*. 2002), in which the caffeine concentration was modified to 5 mM (Wang *et al*. 1991). A portion (10 μl) of the preincubated spermatozoa was introduced into 90 μl of fertilization medium containing about 10 oocytes. The final sperm concentration was adjusted to 1×10^4/ml. Co-incubation of gametes was carried out for 3 or 5 h (standard or prolonged duration) at 39°C under 5% CO_2 and 5% O_2. After co-incubation, spermatozoa attached to the ZP or oolemma were freed from oocytes by gentle pipetting, and the oocytes were

transferred to *in vitro* culture (IVC) medium (IVC-PyrLac) (Kikuchi *et al*. 2002). For examination of the IVF results, inseminated oocytes were cultured subsequently for an additional time at 38.5°C under 5% CO_2 and 5% O_2. They were then fixed with acetic alcohol (1:3), stained with 1% aceto-orcein (Sigma-Aldrich) and examined for sperm penetration parameters using a phase-contrast microscope.

Experimental design

Experiment 1: Effects of pronase treatment of oocytes on sperm penetration

I evaluated the effects of pronase treatment of oocytes on sperm penetration. I prepared ZP-free oocytes as follows. In the first group, mZP− oocytes were incubated for 1 h in IVM medium**.** In the second group, I supplied pZP− 1 h oocytes. Finally, in the third group, I subsequently incubated pZP− 1 h oocytes for an additional 2 h in IVM medium, and these were supplied as "pZP− 3 h" oocytes. The oocytes in the three groups were separately subjected to IVF using a single lot of frozen-thawed epididymal spermatozoa. At 10 h after the initiation of co-incubation of gametes, oocytes in all the groups were fixed, and their sperm penetration parameters were evaluated.

Experiment 2: Effects of ZP on sperm penetration

I evaluated the function of the ZP for *in vitro* sperm penetration during IVF. The ZP+ and ZP− (the same as pZP− 1 h in Experiment 1) oocytes were subjected to IVF using frozen-thawed epididymal spermatozoa from four different boars. At 10 h after the initiation of co-incubation of gametes, oocytes in all groups were fixed and evaluated. The main objective in this experiment was to compare the boar effects on sperm penetration, and to select an appropriate lot for the following experiments to check sperm penetration parameters using ZP− oocytes.

Experiment 3: Evaluation of sperm penetration parameters by time-course monitoring

To clarify whether the ZP and/or oolemma prevents polyspermy, the sperm penetration parameters of ZP+ and ZP− oocytes were examined after addition of a single sperm lot. I evaluated sperm penetration at 1, 2, 3, 4, 5, and 10 h after the initiation of co-incubation of gametes. In the 4, 5, and 10 h groups, after co-culture of the gametes for 3 h, the oocytes were washed gently three times and then incubated in culture medium until fixation. After fixation, I evaluated these oocytes for sperm penetration parameters.

Experiment 4: Evaluation of the possible prevention of sperm penetration by the oolemma

To examine whether or not the oolemma prevented polyspermy, I evaluated the effects of prolongation of the sperm and oocyte co-incubation period from 3 to 5 h on sperm penetration of ZP− oocytes. The ZP− oocytes were divided into two groups depending on the duration of co-incubation: a control group (co-incubation for 3 h) and a prolonged group (co-incubation for 5 h). The oocytes co-incubated with sperm were further incubated without sperm in culture medium before fixation and staining. I fixed the oocytes at 3, 5, and 10 h after the initiation of co-incubation of gametes and then stained and examined them for sperm penetration parameters.

Statistical analysis

The proportions of oocytes penetrated by sperm and the average numbers of penetrated sperm per oocyte were subjected to one-way (Experiment 1) and two-way ANOVA (Experiments 2−4) using the General Linear Models procedures of the Statistical Analysis System (Ver. 9.2, SAS Institute Inc., Cary, NC, USA). Percentage data were arcsine-transformed before the analysis.

RESULTS

Experiment 1

The proportions of sperm that penetrated mZP−, pZP− 1 h, and pZP− 3 h oocytes and the average numbers of penetrated sperm per oocyte are summarized in Fig. 1−1A and 1−1B, respectively. Only oocytes penetrated by sperm were used for calculation of the average number of penetrated sperm per oocyte. After ANOVA, I found no difference between the mZP− group and the other two groups treated with pronase (pZP− 1 h and pZP− 3 h). In the next experiments, I used pZP− 1 h oocytes as zona-free oocytes (hereafter termed ZP− oocytes).

Experiment 2

The combined effects of the ZP present during IVF and utilization of frozen-thawed epididymal spermatozoa from different boars from which sperm were obtained are shown in Fig. 1−2A and 1−2B. The results of ANOVA are shown in Table 1−1. Significant differences in sperm penetration parameters (sperm penetration rates and the average number of penetrated sperm) were detected between ZP+/− groups and also among boars. The proportion of oocytes penetrated by sperm and the average

number of penetrated sperm per oocytes were better in ZP+ oocytes compared with ZP− oocytes. In this experiment, sperm from Boar #3 showed a clear difference in both sperm penetration parameters. In the next experiments (Experiment 3 and 4), as well as in Experiment 1, I therefore used these sperm with the expectation of obtaining clearer results.

Experiment 3

The combined effects of the ZP present during IVF and the period from the initiation of co-incubation of gametes to fixation are shown in Fig. 1−3A and 1−3B. The results of ANOVA are shown in Table 1−2. Significant differences were evident for sperm penetration parameters in both ZP+/− groups and as well as the period from the initiation of co-incubation of gametes. The proportion of oocytes penetrated by sperm and the average number of penetrated sperm per oocyte were better in ZP+ oocytes compared with ZP− oocytes, the sperm penetration parameters increasing with the period from the initiation of co-incubation of gametes to fixation.

Experiment 4

The combined effects of the duration of gamete co-incubation (3 and 5 h) and

period from the initiation of co-incubation of gametes to fixation (3, 5, and 10 h) are shown in Fig. 1−4. The results of ANOVA are shown in Table 1−3. Significant differences were detected in both the duration of gamete co-incubation and period from the initiation of co-incubation of gametes. Longer gamete co-incubation (5 h) made the sperm penetration parameters (the proportion of oocytes penetrated by sperm and the average number of penetrated sperm per oocyte) better compared with the standard period (3 h) when the period from the initiation of co-incubation of gametes to fixation was prolonged to 10 h.

DISCUSSION

To understand the function of the ZP in sperm penetration and blocking of multiple sperm entry, I compared sperm penetration in both ZP+ and ZP− oocytes. Usually, ZP− oocytes can be obtained easily by treatment with pronase (for example, in porcine (Hatanaka *et al*. 1992, Wu *et al*. 2004, Kolbe and Holts 2005), bovine (Fulka *et al*. 1982, Soloy *et al*. 1997), and mouse (Wolf 1976, Zuccotti *et al*. 1991) oocytes). However, I hypothesized that this enzyme treatment might exert some negative effects on sperm penetration, in other words, prevention of polyspermy, in porcine oocytes. Initially, therefore, I evaluated the effects of pronase treatment of oocytes on sperm penetration in Experiment 1. Using mouse oocytes, Yamagata *et al.* (2002) succeeded in removing the ZP using a micromanipulator without treatment with pronase. Thus, in the present study, I also removed the ZP mechanically using a micromanipulator and compared the sperm penetration parameters with those of ZP-denuded oocytes treated with pronase. The results revealed no significant difference in sperm penetration parameters between the pronase-treated group (pZP−) and the group without pronase treatment (mZP−). Furthermore, I checked the possibility of recovery of oocytes or disruption of their integrity after additional culture (1 h vs. 3 h), but no effect was

observed in terms of sperm penetration parameters. Wolf *et al.* (1976) reported that the proportion of sperm penetration of zona-free mouse oocytes prepared by enzymatic treatment (using chymotrypsin and pronase) was less than that of zona-free oocytes prepared mechanically and indicated that this harmful effect was caused by proteolytic alteration of the oolemma upon exposure to these enzymes for a long period (15−30 min). Using mouse oocytes, Zuccotti *et al.* (1991) found that short-term exposure to chymotrypsin for 10 min had little effect on sperm penetration, whereas additional exposure for 15 min reduced sperm penetration significantly. The time required for dissolution of the ZP using pronase is usually much shorter than this. Taken together, it can be suggested that pronase treatment for a shorter period (20−30 sec) has little effect on penetration of sperm into porcine oocytes.

In Experiment 2, the proportion of oocytes penetrated by sperm and the average number of sperm per oocyte (sperm penetration parameters) were significantly lower for ZP− oocytes than for ZP+ oocytes. In the present study, the sperm penetration parameters differed significantly depending upon the boar from which sperm had been obtained. This difference is one of the characteristics of porcine species and has already been reported for frozen-thawed ejaculated and epididymal spermatozoa (Kikuchi et al. 1998, Ikeda *et al*. 2002). Furthermore, from these results, I suggest that

when the ZP is not present, sperm penetration into oocytes cannot be accelerated. The AR plays very important roles in sperm penetration. Acrosome-intact or partially acrosome-reacted sperm can bind to the ZP (Funahashi 2003), and thereafter the AR is induced by the ZP (Berger *et al*. 1989, Wu *et al*. 2004). It is now clear that only acrosome-reacted sperm can pass through the ZP and that after ZP passage they can fuse with the oolemma (Imai *et al*. 1980). On the other hand, in the present study, a certain proportion of ZP− oocytes was also penetrated. Wu *et al.* (2004) reported that 84% of the sperm adherent to ZP-free oocytes lost their acrosome within 1 h after initiation of IVF. Frozen-thawed spermatozoa are already "capacitated" because of cryo-effects on the sperm membrane, so called "cryocapacitation", (Watson 1995, Ikeda *et al*. 2002) and are considered to lose their acrosome spontaneously during incubation in fertilization medium. Therefore, in my experiments, they were able to fuse with the oolemma of ZP− oocytes. However, as mentioned above, a much lower proportion of sperm was able to fuse with the oolemma of ZP− oocytes compared with ZP+ oocytes. This also suggests the importance of the ZP for sperm penetration.

The result of Experiment 2 suggests that the presence of the ZP accelerates sperm penetration, but the result was not enough to discuss the detailed function of the ZP and oolemma for prevention of extra sperm penetration. It seems likely that the

proportion and number of penetrated sperm reach a plateau at a certain time point after the initiation of co-incubation of gametes. In Experiment 3, therefore, to clarify whether polyspermy was prevented by the ZP and/or oolemma, I evaluated sperm penetration parameters with time after the initiation of co-incubation of gametes. The results clearly demonstrated that sperm penetration increased significantly with time after the initiation of co-incubation of gametes. In mammalian oocytes, the zona reaction (zona hardening) is established through a change in the form of the ZP caused by release of cortical granules (Abbott and Ducibella 2001, Sun 2003). In porcine *in vivo* matured oocytes, the zona reaction is induced during fertilization (Kolbe and Holtz. 2005). On the other hand, in *in vitro* matured porcine oocytes, some researchers have reported that the zona reaction is incomplete or delayed (Funahashi *et al*. 2001, Coy *et al*. 2002, Coy and Aviles 2010). Hatanaka *et al.* (1992) reported that zona hardening occurred 12 h after insemination. Therefore, a longer time for complete zona hardening may be required *in vitro* than *in vivo*. It has been reported that the thickness of the ZP and its structure after IVF (especially after release of CG contents) differ between *in vivo* and *in vitro* matured porcine oocytes (Funahashi *et al*. 2001). Furthermore, the resistance of ZP against pronase digestion may similarly differ between *in vivo* and *in vitro* (Funahashi *et al*. 2001, Kolbe and Holtz 2005). It is

possible that these factors are related to failure or delay of zona hardening. In the present study, the results of Experiments 2 and 3 using ZP+ oocytes support these hypotheses. I speculate that the presence or modification of the ZP is not effective for prevention of polyspermy during IVF of *in vitro* matured porcine oocytes.

The results of Experiment 3 indicated that the number of penetrated sperm remained low in ZP− oocytes and did not increase significantly with the duration of IVF. There is a possibility that extra sperm penetration may have been blocked by the oolemma, so called membrane block, after the first sperm penetration. Therefore, in Experiment 4, I prolonged gamete co-incubation from 3 h (standard duration in my laboratory) to 5 h to increase the chance for encounter between the two gametes and examined in detail whether membrane block also occurs during IVF of *in vitro* matured porcine oocytes. Membrane block is the main mechanism for prevention of polyspermy in nonmammalian species (i.e., frogs and several marine invertebrates) (Gould and Stephano 2003). However, in mammalian oocytes, it is considered to be one of the supportive mechanisms of the zona reaction for prevention of polyspermy, but the role of the oolemma has remained unclear (Gardner and Evans 2006). Among mammalian species, the mechanism of membrane block has been examined only in mice (Gardner and Evans 2006, Gardner *et al*. 2007); however, in porcine oocytes, no

studies have investigated this issue. In this study, the proportion of oocytes that were penetrated by sperm and the average number of penetrated sperm per oocyte were significantly higher in the prolonged IVF group than those in the control group. This suggests that sperm penetration may increase if the opportunity for oocytes to encounter sperm is prolonged. On the other hand, membrane block in mouse oocytes is reported to be functional (Gardner and Evans 2006). McAvey *et al.* (2002) reported that when ZP-free mouse oocytes were subjected to IVF, the number of sperm that fused with oocytes reached a plateau at 2 h after the initiation of co-incubation of gametes. Other studies using ZP-free oocytes of the mouse, hamster, and human have also shown reduction of the binding and fusion abilities of the oolemma after insemination (Zuccotti *et al*. 1991, Horvath *et al*. 1993, Sengoku *et al*. 1995). Elevation of intracellular calcium levels (corresponding to oocyte activation) is important for the establishment of membrane block in mouse oocytes (McAvey *et al.* 2002). It has not been sure if there is a similar mechanism for membrane block in porcine oocytes because there has been no report about this phenomenon. My results, however, suggest that the oolemma is not effective for preventing polyspermic penetration of ZP− oocytes or that complete membrane block is not involved in the porcine IVF system.

In conclusion, the ZP and oolemma are not competent factors for prevention of polyspermy, at least in the present porcine IVF system using in our laboratory. However, the presence of the ZP accelerates sperm penetration into the ooplasm in pigs.

FIGURE AND TABLES

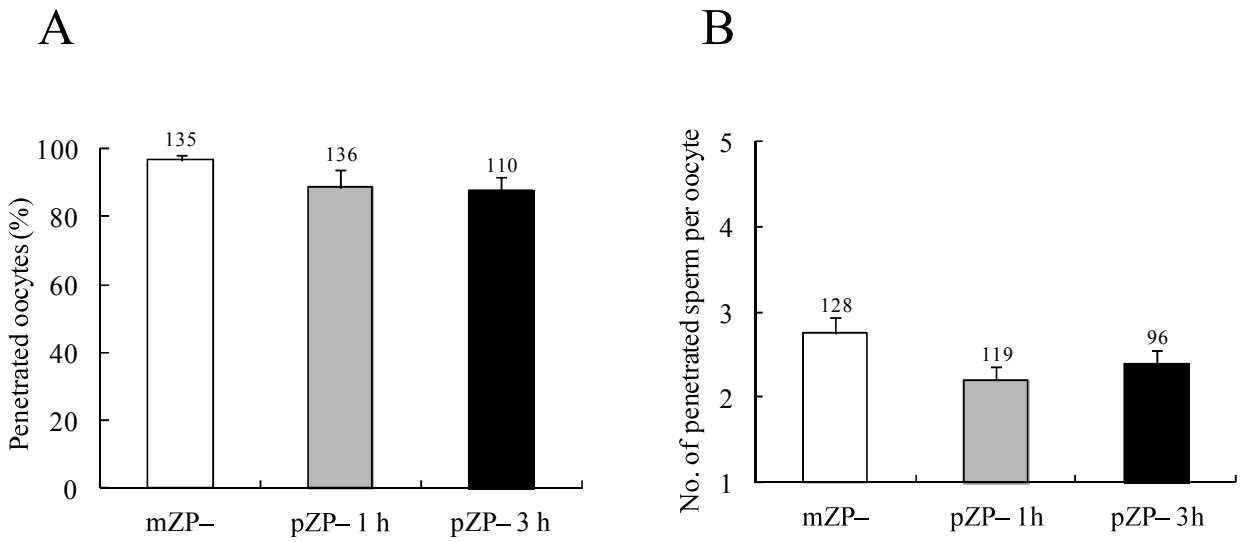

Fig. 1−1 The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) in each of the treatment groups fixed at 10 h after the initiation of co-incubation of gametes. mZP− oocytes were denuded of the zona pellucida without pronase treatment. The other two groups, pZP− 1 h and pZP− 3 h oocytes, were treated with pronase to remove the ZP and then cultured for 1 h and 3 h, respectively. ANOVA demonstrated no differences among the three groups. Replicated trials were performed seven times. Numbers above the bars indicate total numbers of oocytes used in the experimental groups. Means ± SEM are presented.

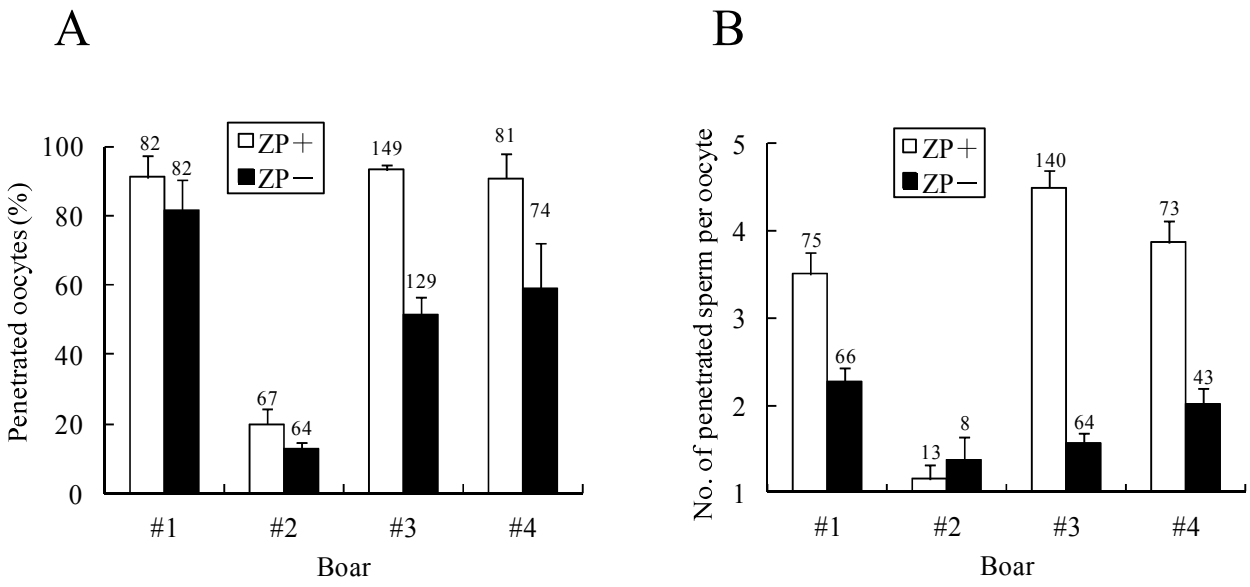

Fig. 1−2 The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) in ZP+ and the ZP− oocytes fixed at 10 h after the initiation of co-incubation of gametes. Frozen-thawed epididymal spermatozoa from 4 different boars were used (Boars #1−4). The results of ANOVA are shown in Table 1−1. When the ZP was present, sperm penetration was significantly accelerated. Replicated trials were repeated three times for each group. Numbers above the bars indicate total numbers of oocytes used in the experimental groups. Means ± SEM are presented.

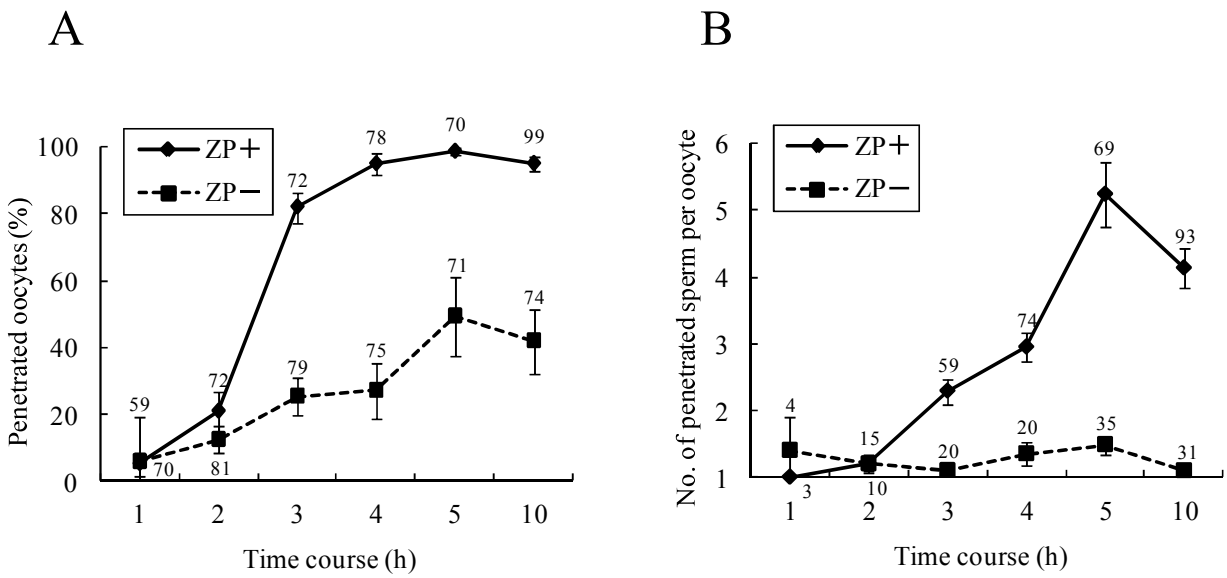

Fig. 1−3 The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) for ZP+ and ZP− oocytes at 1, 2, 3, 4, 5, and 10 h after the initiation of co-incubation of gametes. I used frozen-thawed epididymal spermatozoa from one lot (Boar #3 in Fig. 1−2), for which a marked difference in sperm penetration was observed between the ZP+ and ZP− oocytes used in experiment 2. The results of ANOVA are shown in Table 1−2. Numbers above or under the plots indicate total numbers of oocytes used in the experimental groups. Replicated trials were performed five times. Means ± SEM are presented.

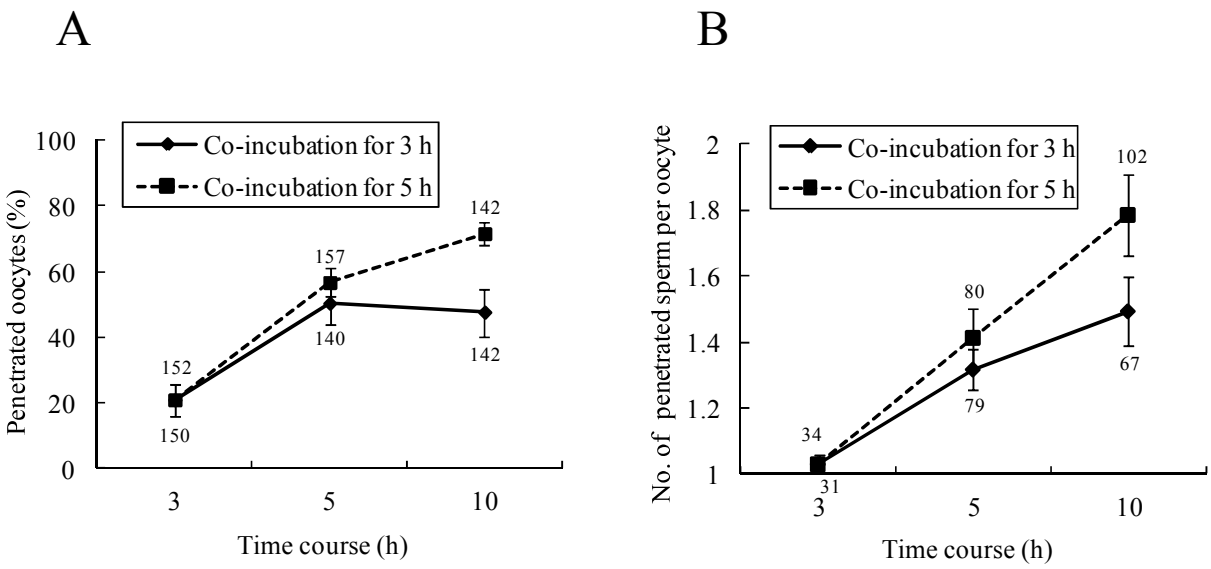

Fig. 1−4 The proportion of penetrated ZP− oocytes (A) and the average number of penetrated sperm per oocyte (B) in the control (co-incubation for 3 h) and prolonged co-incubation groups (co-incubation for 5 h) at 3, 5, and 10 h after the initiation of co-incubation of gametes. I used the same frozen-thawed epididymal spermatozoa (Boar #3 in Fig. 1−2). The results of ANOVA are shown in Table 1−3. Numbers above or under the plots indicate total oocyte numbers used for experimental groups. Experiments were repeated five times. Means ± SEM are presented.

Table 1−1. ANOVA of sperm penetration parameters according to presence of the zona pellucida (ZP) and sperm origin from different boars.

Source	% of penetrated oocytes			No. of penetrated sperm		
	df	Mean square	F value	*df*	Mean square	F value
Presence of ZP	1	0.925	17.43[a]	1	458.330	124.42[a]
Boar	3	1.050	19.79[a]	3	31.428	8.53[a]
Interaction between ZP and Boar	3	0.119	2.24	3	27.830	7.55 [a]

ZP: intact (ZP+) or removed (ZP−). Boar: 4 boars. *Df*: degree of freedom

[a] $P < 0.01$

Table 1−2. ANOVA of sperm penetration parameters according to presence of the zona pellucida (ZP) and period from the initiation of co-incubation of gametes to fixation.

Source	% of penetrated oocytes			No. of penetrated sperm		
	df	Mean square	F value	df	Mean square	F value
Presence of ZP	1	6.996	137.12[a]	1	469.009	86.62[a]
Period from the initiation of co-incubation of gametes	5	1.741	34.12[a]	5	64.144	11.85[a]
Interaction between ZP and the initiation of co-incubation of gametes	5	0.745	14.60[a]	5	26.452	4.89[a]

ZP: intact (ZP+) or removed (ZP−), Period from the initiation of co-incubation of gametes to fixation: 1, 2, 3, 4, 5, and 10 h. *df:* degree of freedom

[a] $P < 0.01$

Table 1−3. ANOVA of sperm penetration parameters into ZP-free oocytes according to duration of gamete co-incubation and period from the initiation of co-incubation of gametes to fixation.

Source	% of penetrated oocytes			No. of penetrated sperm		
	df	Mean square	F value	*df*	Mean square	F value
Duration of gamete co-incubation	1	0.114	5.96[a]	1	4.511	6.50[a]
Period from the initiation of co-incubation of gametes	2	0.549	28.68[b]	2	10.869	15.67[b]
Interaction between co-incubation and the initiation of co-incubation of gametes	2	0.060	3.14	2	1.336	1.93

Duration of gamete co-incubation: 3 and 5 h. Period from the initiation of co-incubation of gametes to fixation: 3, 5, and 10 h. *df:* degree of freedom

[a] $P < 0.05$; [b] $P < 0.01$

ARTICLE 2

Roles of the Zona Pellucida and

Functional Exposure of the Sperm-egg Fusion Factor “IZUMO”

ABSTRACT

The zona pellucida (ZP) is considered to play important roles in the prevention of polyspermy in mammalian oocytes. In pigs, however, I have shown that the presence of the ZP accelerates sperm penetration into the ooplasm during *in vitro* fertilization (IVF) (ARTICLE 1). The functions of the ZP that are responsible for this result have remained unclear. The sperm possess ZP adhesion molecules. Furthermore, the acrosome reaction, being considered to be induced by the ZP, is necessary for sperm to fuse with oolemma. It is considered that these factors may accelerate sperm penetration in ZP+ oocytes. In the present study, firstly, I investigated the effects of the ZP on sperm binding and its acrosomal status. I evaluated the numbers of sperm bound to the ZP in ZP+ oocytes and oolemma in ZP− oocytes. Acrosomal statuses of these binding sperm were also evaluated. Furthermore, I evaluated the numbers and acrosomal statuses of sperm presenting in the ZP and perivitelline space (PVS) using ZP+ and ZP− oocytes. The average number of sperm bound to ZP+ oocytes was significantly higher than that bound to ZP− oocytes. The average number of sperm bound to oolemma and lost their acrosome was 6.15−11.28 per oocyte in ZP− oocytes. More sperm bound to the ZP than to the oolemma. The average number of sperm present in

the PVS was 0.44−0.51 per oocyte, and all sperm had lost their acrosomes. I found that the sperm in the PVS, in other words, the sperm passing through the ZP fuse with oolemma with high efficiency. It may be considered that the sperm are induced some kind of factors involved in sperm-oolemma fusion by passing through the ZP. Recently, IZUMO, one of the membrane proteins on sperm, was discovered as critical factor for sperm-oolemma fusion in mice and human. So in the second experiment, I hypothesized that IZUMO on the sperm membrane gives grate effect on membrane fusion. When sperm in the ZP+ oocytes were applied to immunological detection of IZUMO during IVF, the proportion of sperm that were immunopositive for anti-IZUMO antibody was significantly higher after they were passing or had passed through the ZP. Next, I performed addition of anti-IZUMO antibody to the fertilization medium, it reveals the significant inhibition on the penetration of sperm into ZP− oocytes. Finally, I investigated whether synthesis of IZUMO depends on the association of ZP during IVF using two kinds of protein synthesis inhibitors, chloramphenicol (CP) and cycloheximide (CH), which inhibit mRNA translation (protein synthesis) completely in eukaryotic cells including sperm I collected matured oocytes and performed IVF using fertilization medium with CP and CH and compared proportion of sperm that were immunopositive for anti-IZUMO antibody. There was

no significant difference among experimental groups. These results suggest that, in pigs, the ZP induces the acrosome reaction, which is associated with the functional exposure of IZUMO, resulting in completion of fertilization. IZUMO may not be synthesized during IVF and it may be considered that IZUMO undergoes post-translational modification, changing their location, or some other modifications resulting in exposure of IZUMO during passing through the ZP.

INTRODUCTION

In porcine oocytes, polyspermy occurs at high frequency and is considered to be an obstacle to efficient *in vitro* production of normal embryos (Funahashi 2003, Nagai *et al.* 2006, Kikuchi *et al*. 2009). I have been focusing on the roles of the ZP in sperm penetration into the ooplasm during IVF using *in vitro* matured porcine ZP-intact and ZP-free oocytes, and have shown that the presence of the ZP accelerates sperm penetration (ARTICLE 1). In mammalian oocytes, the zona reaction (zona hardening) occurs through a change in the form of the ZP caused by release of cortical granules, and this prevents the penetration of extra sperm (Abbott and Ducibella 2001, Sun 2003). However, in *in vitro* matured porcine oocytes, some researchers have shown that the zona reaction for prevention of polyspermy is incomplete or delayed (Hatanaka *et al.* 1992, Funahashi *et al.* 2001, Coy *et al.* 2002, Coy and Aviles 2010). My previous results (ARTICLE 1) have supported these reports, but the reasons why the ZP accelerates sperm penetration, and the functions of the ZP that are responsible for these phenomena, have remained unclear.

The AR plays very important roles in sperm penetration. Sperm in which the acrosome is intact or partially reacted can bind to the ZP (Funahashi 2003), and

thereafter the complete AR is induced by the ZP (Berger *et al.* 1989). It has been considered that only acrosome-reacted sperm can fuse with the oolemma (Imai *et al.* 1980). It has also been suggested that, during the AR induced by the ZP, one or several important factors may participate in successful sperm penetration into the ooplasm. Sperm possess ZP adhesion molecules such as zonadhesin (Hardy and Garbers 1994, Hardy and Garbers 1995, Hickox *et al.* 2001, Bi *et al.* 2003), β-1,4-galactosyltransferase (Rebeiz & Miller 1999), and proacrosin/acrosin (Yonezawa *et al.* 1995a). It is possible that these factors accelerate the binding of sperm to oocytes, thus accelerating sperm penetration into the ooplasm. On the other hand, in ZP-intact oocytes, only sperm that have passed through the ZP and are present in the PVS can fuse with the oolemma. The presence of sperm in the PVS is necessary for successful fertilization. In the ARTICLE 2, I evaluated the numbers and acrosome statuses of sperm binding to the ZP in ZP-intact oocytes, to the oolemma in ZP-free oocytes, and being present in the PVS in ZP-intact oocytes.

It is possible that the ZP may accelerate functions of some factors involved in sperm-oolemma fusion. One such factor is Izumo, which was initially reported as a sperm-egg fusion factor in mice and humans, belonging to the mammalian immunoglobulin protein family (Inoue *et al.* 2005). As described before, this type of

Izumo, “Izumo1”, was a member of the Izumo multiprotein family in mice (Ellerman *et al.* 2009). Other members are Izumo2−4, whose N-terminal domains showed significant homology to that of the Izumo1. Sperm from *Izumo*-knockout mice are able to pass through the ZP, but cannot fuse with the oolemma (Inoue *et al.* 2005). The expression of IZUMO has also been analyzed in porcine species, and shown to be specific to boar sperm (Kim *et al.* 2012). In humans, the relationship between IZUMO and infertility has been studied, and anti-IZUMO antibodies have been found in serum samples from immunoinfertile women (Clark and Naz 2013). I hypothesize that the presence of IZUMO may be correlated with successful membrane fusion and completion of sperm penetration into the ooplasm, resulting in differences in the manner of sperm penetration between ZP-intact and ZP-free oocytes.

It is widely accepted that mature spermatozoa are translationally silent. However, Gur and Breitbart (2006) reported that nuclear genes are expressed to produce some proteins in human, bovine, mouse, and rat spermatozoa during capacitation (for example, human; protein kinase C, epidermal growth factor receptor protein, bovine; protein kinase C, AKAP110, mouse; CatSper, catalytic subunit of protein kinase A, rat; Na-K-ATPase). In bovine spermatozoa, these proteins are synthesized within 60 min under the conditions to induced capacitation. They investigated the synthesis of protein

using two kinds of protein synthesis inhibitor, chloramphenicol (CP) and cycloheximide (CH). Isolated mitochondria are known to be capable of protein synthesis independently of the cytoplasmic ribosomes (Mclean *et al*. 1958). CP and CH inhibits 55S mitochondrial ribosomes and 80S cytoplasmic ribosomes, respectively, resulting in complete inhibition of mRNA translation (Ashwell and Work 1970). In spermatozoa, Gur and Breitbart (2006) showed that the synthesis of some proteins involved in capacitation proved sensitive to the CP, but insensitive to the CH in humans, mice, cows, and rats. It indicates that matured sperm can undergo protein synthesis. In the present study, I used CP and CH to evaluate if synthesis of IZUMO may occur or not during IVF.

In ARTICLE 2, I evaluated the roles of IZUMO in penetration of sperm into the ooplasm during IVF in pigs by analyzing the presence of IZUMO on the sperm using anti-IZUMO antibody. Furthermore, I investigate if synthesis of IZUMO may occur during IVF using two kinds of protein synthesis inhibitors, CP and CH.

MATERIALS AND METHODS

Preparation of ZP-intact and ZP-free oocytes

After in vitro maturation of COCs, cumulus cells were removed from the COCs. Denuded oocytes with the first polar body were harvested under a stereomicroscope and used as *in vitro* matured and ZP-intact oocytes (ZP+ oocytes). These matured oocytes were exposed to 0.5% (w/v) pronase in PBS for 20−30 s (Peura & Vajta 2003). Oocytes with an expanded and deformed ZP were then transferred to M199 without pronase and freed completely from the ZP by gentle pipetting. After 1 h of incubation in maturation medium at 39°C under 5% CO_2 and 5% O_2, these ZP-free oocytes, termed "ZP− oocytes", were used for further experiments.

Sperm-oocyte binding assay and observation of sperm in the PVS

The sperm-oocyte binding assay of ZP+ oocytes was performed at 1, 3, and 5 h after the initiation of gamete co-incubation (termed as 1, 3, and 5 h groups, respectively) essentially as described previously (Noguchi *et al.* 1992, Yonezawa *et al.* 1995b). The sperm binding assay for ZP− oocytes was conducted on the basis of this method. First, I performed IVF by adjusting the final sperm concentration to 1 ×

10^4/ml, and co-incubation of gametes was carried out for 1 h (1 h group) or 3 h (3 h group and 5 h group). At 1, 3, and 5 h after the initiation of gamete co-incubation, oocytes were washed by transfer to 30 μl drops of PBS containing 0.5% BSA in order to remove loosely bound and unbound sperm, employing a pipette with a 180−200 μm bore size, which just slightly exceeds the diameter of a porcine oocyte. In 1 h and 3 h groups, oocytes were washed ten times. At 3 h after the initiation of co-incubation of gametes, 5 h group-oocytes were washed three times in IVC-PyrLac and transferred to fresh IVC-PyrLac in order to be cultured for additional 2 h. After 2 h, these oocytes were washed six times (the washing was performed also ten times in total in 5 h group). Some ZP+ oocytes were fixed with 3% glutaraldehyde for 30 min at room temperature. The sperm heads bound to the oocytes were stained with 50 μg/ml bisBenzimide H 33342 (Hoechst 33342; Calbiochem Corp., La Jolla, CA, USA) in PBS and the numbers of sperm bound to each oocyte were counted using a fluorescence microscope (BX-51, Olympus, Tokyo, Japan) with a WU filter (Olympus). The other ZP+ oocytes were incubated in 1 mg/ml pronase in PBS for approximately 2 min. After slight expansion of the PVS, they were stained with 100 μg/ml fluorescein isothiocyanate-conjugated peanut lectin (FITC-PNA; L7381, Sigma-Aldrich) in PBS and observed with a WIB filter (Olympus). This procedure allows evaluation of the

acrosomal status of sperm present in the PVS within an expanded ZP. On the other hand, the inseminated and washed ZP− oocytes were subjected to the binding assay after staining with Hoechst 33342 and also with FITC-PNA, and then the acrosomal status of sperm binding to the oolemma was evaluated.

Immunostaining for IZUMO

I performed IVF using both ZP+ and ZP− oocytes, adjusting the final sperm concentration to 1×10^5 /ml. For ZP+ oocytes at 3 h after the initiation of co-incubation of gametes, I removed sperm binding to the surface of the ZP by pipetting. I then treated the oocytes with hypertonic PBS solution (400 mOsmol/kg) for 10 min to shrink the ooplasm and to expand the PVS. ZP− oocytes, on the other hand, were washed ten times in 30-μl drops of PBS containing 0.5% BSA for removing extra sperm bound to the oolemma. The oocytes with sperm in both groups were fixed with 3.7% paraformaldehyde in PBS for 30 min at 4°C. They were then blocked in 1% skim milk (Snow Brand Milk Products Co. Ltd., Sapporo, Japan) in PBS for 3 h at 37°C, and incubated overnight at 4°C with 0.25 μg/ml (1:800) anti-IZUMO antibody (anti-human IZUMO1 antibody raised in goat, sc-79543; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS supplemented with 0.1% Tween 20

(Sigma-Aldrich) (T-PBS). Washes were performed three times in T-PBS, followed by 1 h incubation at room temperature with 1:800 rhodamine-conjugated donkey anti-goat antibody (AP180R; Merck Millipore, Inc., Billerica, MA, USA) as the secondary antibody. After several washings in T-PBS, oocytes were stained with FITC-PNA (100 μg/ml in PBS) for 15 min at 37°C, then counterstained with Hoechst 33342 and mounted in 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich) in a mixture of 90% glycerol and 10% PBS. Then, ZP− oocytes were crushed under a cover glass to allow them to be seen without any interfering bright fluorescence from oocytes. All fluorescence images were obtained using a CCD camera (Cool SNAP cf; Photometrics, Tucson, AZ, USA) equipped with WU, WIB, and WIG filters (Olympus). In the present study I used the term “IZUMO+” sperm for sperm that had lost their acrosomes and were immunopositive for IZUMO.

IVF with anti-IZUMO antibody

I prepared ZP− oocytes and performed IVF using Pig-FM supplemented with the anti-IZUMO antibody. The procedure used for IVF was the same as that described above. In brief, frozen-thawed epididymal spermatozoa were preincubated for 15 min in Medium 199 with Earl’s salts adjusted to pH 7.8. Oocytes were transferred to

Pig-FM that had been supplemented with the anti-IZUMO antibody. A portion (10 μl) of the preincubated spermatozoa was added to 90 μl of fertilization medium containing about 10 oocytes. The final concentration of the antibody in the fertilization medium was set as 0 (control), 0.25 or 0.5 μg/ml, and the final sperm concentration was adjusted to 1×10^4/ml. After co-incubation of gametes for 3 h, the oocytes were transferred to IVC-PyrLac. For examination of the IVF results, the inseminated oocytes were subsequently cultured for 7 h at 38.5°C under 5% CO_2 and 5% O_2. They were then fixed, stained and examined for sperm penetration parameters using a phase-contrast microscope.

IVF with protein synthesis inhibitors

I prepared ZP+ oocytes and performed IVF using Pig-FM supplemented with CP (0.1 mg/ml) and CH (1 mg/ml) (Gur and Breitbart 2006). The procedure used for IVF was the same as that described above. In brief, frozen-thawed epididymal spermatozoa were preincubated for 15 min in Medium 199 with Earl's salts adjusted to pH 7.8. Oocytes were transferred to Pig-FM that had been supplemented with CP (Sigma-Aldrich,) and CH (Sigma-Aldrich). A portion (10 μl) of the preincubated spermatozoa was added to 90 μl of fertilization medium containing about 10 oocytes

and gametes were co-incubated for 3 h. The final sperm concentration was adjusted to 1×10^5 /ml.

Experimental design

Experiment 1: Observation of sperm binding to oocytes and passage through the ZP

I evaluated ZP+ and ZP− oocytes for the numbers of sperm binding to the ZP and oolemma, respectively. In the ZP− oocytes, I also evaluated the acrosomal status of the binding sperm. I performed a binding assay and double staining with Hoechst 33342 and FITC-PNA for observation of the number and acrosomal status of sperm binding to ZP+ oocytes and ZP− oocytes at 1, 3, and 5 h after the initiation of co-incubation of gametes. In the ZP+ oocytes, “binding sperm” were defined as sperm both binding to the surface of the ZP and passing through it (i.e. part or all of the sperm head was located in the area of the ZP). In ZP− oocytes, all sperm bound to the oolemma without fusion to the membrane (i.e. the sperm head retained its original shape and size). Furthermore, I also checked for the presence of sperm in the PVS in ZP+ oocytes, and evaluated their number and acrosome status.

Experiment 2: Evaluation of anti-IZUMO antibody specificity and presence of IZUMO

in sperm

Prior to immunostaining of IVF oocytes, I checked the specificity of the first antibody in sperm smear preparations. The spermatozoa were incubated for 2 h in fertilization medium, then washed in PBS and fixed with 3.7% paraformaldehyde in PBS. After washing in PBS and air drying on glass slides, they were blocked with 1% skim milk in PBS and incubated overnight at 4°C with the first antibody. Washes were performed in T-PBS, followed by a 1 h-incubation at room temperature with the secondary antibody. After several washings in T-PBS, oocytes were stained with FITC-PNA. I then evaluated the acrosome status and detected the localization of IZUMO by FITC-PNA staining and immunostaining using anti-IZUMO antibody, respectively. In some preparations, I did not apply the first antibody in order to check for any non-specific reaction. Finally, I detected the nucleus, acrosomal status and localization of IZUMO in sperm associated with both ZP+ and ZP− oocytes at 3 h after the initiation of co-incubation of gametes by staining with Hoechst 33342, FITC-PNA and immunostaining, respectively.

Experiment 3: Effects of addition of anti-IZUMO antibody to fertilization medium on sperm penetration

To evaluate the role(s) of IZUMO in sperm penetration, I performed IVF of ZP− oocytes using Pig-FM containing the anti-IZUMO antibody (0 μg/ml as a control, 0.25, or 5 μg/ml). Some oocytes were fixed at 1, 3, and 5 h after the initiation of co-incubation of gametes and subjected to the binding assay in each experimental group. The other oocytes were inseminated for 3 h and then further cultured for up to 10 h and fixed. They were then evaluated for sperm penetration parameters.

Experiment 4: IVF with protein synthesis inhibitors and immnostaining for IZUMO

To evaluate whether IZUMO is synthesized in spermatozoa during IVF, I performed IVF using Pig-FM containing CP and CH, the mitochondrial and cytoplasmic protein synthesis inhibitor, respectively. After co-incubation of gametes for 3 h, I performed immunostaining for IZUMO and evaluated the proportion of IZUMO+ sperm.

Statistical analysis

Differences in the average number of sperm binding to oocytes were subjected to two-way ANOVA (Experiments 1 and 2). The proportions of oocytes penetrated by sperm, the average numbers of sperm that had penetrated per oocyte (Experiment 2),

and the proportions of sperm that lacked an acrosome and were immunopositive for IZUMO (Experiment 3 and 4) were subjected to one-way ANOVA. These statistical analyses were performed using the General Linear Models procedures of the Statistical Analysis System. Percentage data were arcsine-transformed before the analysis.

RESULTS

Experiment 1

The results of the binding assay for evaluating the number of sperm that had bound to the ZP in ZP+ oocytes and to the oolemma in ZP− oocytes are shown in Fig. 2−1A. The results of ANOVA are shown in Table 2−1. There were significant differences in the number of binding sperm according to the presence of the ZP; more sperm bound to ZP+ oocytes than to ZP− oocytes. However, the period from the initiation of co-incubation of gametes to the end of culture (1, 3, and 5 h) did not affect sperm binding to the ZP or oolemma. The average numbers of sperm present in the PVS of ZP+ oocytes were constant (0.44−0.51 sperm/oocyte) during the period (Fig. 2−1B), and all of them lost their acrosome. The numbers of sperm that had lost their acrosome and become bound to ZP− oocytes are shown in Fig. 2−1C. The number of sperm significantly increased at 3 h after the initiation of co-incubation of gametes, and reached a plateau towards the end of *in vitro* culture (5 h). The average number of binding sperm was 6.15−11.28 per oocyte.

Experiment 2

Sperm smear preparations were stained with FITC-PNA (Fig. 2−2A) and anti-IZUMO antibody (Fig. 2−2B). Some sperm appeared to have lost their acrosomes spontaneously, and were FITC-PNA-negative. Such sperm were divisible into two groups: IZUMO+ and IZUMO−. On the other hand, all acrosome-intact (FITC-PNA-positive) sperm were recognized as IZUMO−. When immunostaining was performed without the first antibody, no signals were detected in any (either acrosome-lost or -intact) sperm (Fig. 2−2C).

ZP+ oocytes were stained with Hoechst-33342 (Fig. 2−3A), FITC-PNA (Fig. 2−3B), and anti-IZUMO antibody (Fig. 2−3C). These results clearly indicated that sperm in the PVS, whose heads were stained with Hoechst-33342, lacked the acrosome (FITC-PNA-negative). Both IZUMO+ and IZUMO− sperm prepared from ZP− oocytes were detectable by the same staining procedures (data not shown). The acrosome statuses of sperm that were passing through the ZP (sperm in the ZP) and sperm that had passed through the ZP (sperm in the PVS) of ZP+ oocytes, and also those bound to ZP− oocytes, are summarized in Fig. 2−4A. All sperm in the ZP and PVS of ZP+ oocytes had lost their acrosome, compared with only about 70% of the sperm on ZP− oocytes. Among these sperm that had lost their acrosome, the proportions of IZUMO+ sperm in the ZP and PVS were significantly higher in ZP+

oocytes than that in ZP− oocytes (Fig. 2−4B).

Experiment 3

The results of binding assay during IVF using fertilization medium supplemented with anti-IZUMO antibody are shown in Fig. 2−5A. The data were subjected to ANOVA and the results are shown in Table 2−2. No significant differences were evident among the concentrations of anti-IZUMO antibody employed. However, the number of binding sperm increased as the period of co-incubation of gametes was prolonged. When fertilization parameters were evaluated, the percentage of oocytes that had been penetrated by sperm significantly decreased in medium that had been supplemented with the antibody (Fig. 2−5B), and the average number of penetrated sperm per oocyte decreased significantly as the concentration of the antibody was increased from 0 to 0.5 μg/ml (Fig. 2−5C).

Experiment 4

All sperm that were passing through the ZP (sperm in the ZP) and had passed through the ZP (sperm in the PVS) of ZP+ oocytes had lost their acrosome in control, CP treated, and CH treated group (data not shown). In the sperm that had lost their

acrosome, the proportions of IZUMO+ sperm in the ZP and PVS are not different among three experimental groups (Fig. 2−6).

DISCUSSION

In ARTICLE 1, I had shown that the proportion of oocytes penetrated by sperm and the average number of sperm per oocyte for ZP− oocytes were significantly lower than those for ZP+ oocytes, suggesting that the presence of the ZP accelerated the penetration of sperm into the ooplasm. I considered that one possible explanation for this may have been due to a difference in the ability of sperm to bind to ZP+ and ZP− oocytes. Braundmeier *et al.* (2004) reported that artificial insemination with sperm showing high zona-binding ability produced larger litters. The results of my present binding assay suggested that higher numbers of sperm became bound to ZP+ oocytes than to ZP− oocytes. I consider that this was due to the presence of adhesion molecules against the ZP in sperm, such as zonadhesin (Hardy and Garbers 1994, 1995, Hickox *et al.* 2001, Bi *et al.* 2003), β-1,4-galactosyltransferase (Rebeiz and Miller 1999), and proacrosin/acrosin (Yonezawa *et al.* 1995a). The removal of the ZP may reduce sperm binding to ZP− oocytes. The present results suggest that the ZP increases the opportunity of oocyte penetration by sperm, thus accelerating sperm penetration into the ooplasm. However, before completion of penetration into the ooplasm sperm must bind to the oolemma. It has been considered that sperm present in the PVS of ZP+

oocytes are able to bind to the oolemma. Therefore, in the present study, it was necessary to investigate if sperm present in the PVS during and after insemination can bind to the oolemma. On the other hand, in ZP− oocytes, as the ZP had been removed completely, all sperm observed on the surface were considered to have bound to the oolemma, and then some of them should subsequently participate in sperm-oolemma fusion.

One of the important factors determining sperm-oolemma fusion is whether sperm binding to the oolemma have lost their acrosome. A specific fluorescent stain, FITC-PNA, is known to detect the sperm acrosome and has been used in studies of porcine species (Fazeli *et al.* 1997). Therefore, I performed staining with both FITC-PNA and Hoechst 33342 in order to evaluate the acrosomal status and nucleus, respectively, of sperm present in the PVS of ZP+ oocytes, and also those binding to surface (oolemma) of ZP− oocytes. The average number of sperm that had lost their acrosome and bound to the surface of the oolemma in ZP− oocytes (6.15−11.28 per oocyte) (Fig. 2−1C) was apparently higher than that of sperm present in the PVS of ZP+ oocytes (0.44−0.51 sperm/oocyte) (Fig. 2−1B). However, the average number of sperm that successfully penetrated the ooplasm was higher in ZP+ oocytes than in ZP− oocytes (ARTICLE 1). These results suggest that sperm passing through the ZP are

able to penetrate the oolemma with high efficiency.

It is considered that, in ZP− oocytes, sperm that penetrated the ooplasm had lost their acrosome, even though they did not pass through the ZP. The proportion of sperm lacking an acrosome varies according to preservation or incubation conditions. Some fresh or frozen-thawed boar spermatozoa lose their acrosome spontaneously during incubation (Coy *et al.* 2002, Han *et al.* 2006, Miah *et al.* 2006). However, as mentioned above, penetration efficiency is lower for sperm that have lost their acrosome spontaneously (in ZP− oocytes) than that for those lacking an acrosome that are passing or have passed through the ZP (in ZP+ oocytes). I assume that certain mechanism(s) may be important for induction of sperm penetration by passage through the ZP.

Inoue *et al.* (2005) were the first to report Izumo as a critical factor for sperm-oolemma fusion in mice, Izumo having been originally characterized as the antigen recognized by a monoclonal antibody, OBF13, raised against mouse sperm (Okabe *et al.* 1987). Kim *et al.* (2012) subsequently cloned and characterized the porcine *IZUMO1* gene, raised an anti-porcine IZUMO1 antibody, and analyzed the expression of IZUMO1 in porcine species. Furthermore, they found that IZUMO1 is well conserved across species (pig, crab-eating macaque, mouse, and bull). In the

present study, I used a commercially available anti-IZUMO1 antibody raised against the human *IZUMO1* sequence; however, its specificity for boar spermatozoa was confirmed in Experiment 2 (Fig. 2−2A−C) and I observed IZUMO+ sperm present in the PVS of ZP+ oocytes (Fig. 2−3A−C). It is considered that sperm lacking an acrosome that had bound to the surface of ZP− oocytes had lost their acrosome spontaneously. It was also notable that sperm in the ZP and PVS of ZP+ oocytes had all lost their acrosome, and that the proportion of IZUMO+ sperm in ZP+ oocytes was significantly higher than that in ZP− oocytes (Fig. 2−4A, B). In mice, it has been reported that Izumo1 became detectable on a large proportion of sperm that had suffered spontaneous loss of the acrosome (Yamashita *et al.* 2007). In the present study, however, the percentage of sperm that had lost their acrosome spontaneously and were immunoreactive for IZUMO was very low. On the other hand, IZUMO became detectable on sperm that were passing and had passed through the ZP with high efficiency, suggesting that the ZP promotes the immunological detection of IZUMO in pigs. Further investigation of the effects of IZUMO on fertilization in mammalian species, including pigs, will be necessary.

In Experiment 3, I evaluated the roles of IZUMO in sperm penetration during porcine IVF. When I performed IVF using fertilization medium that had been

supplemented with anti-IZUMO antibody, sperm penetration into the ooplasm was significantly inhibited (Fig. 2−5B, C), suggesting that IZUMO is important for completion of sperm penetration. Furthermore, my binding assay showed that supplementation with anti-IZUMO antibody was unable to inhibit sperm-oolemma binding (Fig. 2−5A). Inoue *et al.* (2005) suggested that although sperm collected from *Izumo*-knockout male mice were able to bind to the ZP and oolemma of ZP-free mouse oocytes, fertilization failed. My present findings also suggest that IZUMO may not participate in sperm-oolemma binding in porcine species, but rather plays a role in sperm membrane-oolemma fusion for completion of fertilization.

In Experiment 3, I had shown that, after the sperm were passing or had passed through the ZP, IZUMO was exposed efficiency. It is also indicated that the sperm became immunopositive for IZUMO in a short time during the sperm contact to and passing through the ZP (0 h to 3 h). Gur and Breitbart (2006) used protein synthesis inhibitors, CP and CH, and suggest the possibility that the mature spermatozoa are able to synthesize some proteins during incubation in capacitation media *in vitro*. IZUMO is also one of the proteins in spermatozoa, so there may be a possibility that the synthesis of IZUMO occurs during IVF and also inhibited by CP or CH. In Experiment 4, I investigated whether IZUMO is synthesized in sperm during passing through the ZP.

Observation of IZUMO+ sperm after IVF with CP and CH indicates that IZUMO may not be synthesized during IVF. Miranda *et al*. (2009) investigated the localization of the proteins in the sperm and their behavior during capacitation and AR in mice. They reported that some of these proteins modify their immunofluorescence pattern, and IZUMO changes its location after the AR. In intact sperm, IZUMO was restricted to the dorsal portion of the sperm head (observed after permeabilization). After the AR, it districted to a new region, adjacent regions to equatorial segment. In pigs, IZUMO may be also redistributed and result in immunopositive after normal AR. Another possibility is that IZUMO becomes immunopositive after post-translational modification. Baker *et al*. (2012) suggest that rat IZUMO1 undergoes post-translational modification during epididymal maturation. The ZP may possibly induce post-translational modification of IZUMO. The result in my study and these reports may indicate that IZUMO have already been synthesized in mature spermatozoa, and become detectable after some kind of modification. Therefore, in my study, I describe immunological detection of IZUMO as “exposure” in order to distinguish it from “expression”, generally meaning nuclear gene translation as protein. Moreover, further studies are needed to clarify the mechanisms.

In conclusion, the ZP induces the AR and functional exposure of IZUMO, thus

facilitating successful fertilization in pigs. Porcine IZUMO may not be synthesized during IVF and it may be considered that IZUMO undergoes post-translational modification, changing their location, or some other modifications resulting in exposure of IZUMO during passing through the ZP.

FIGURE AND TABLES

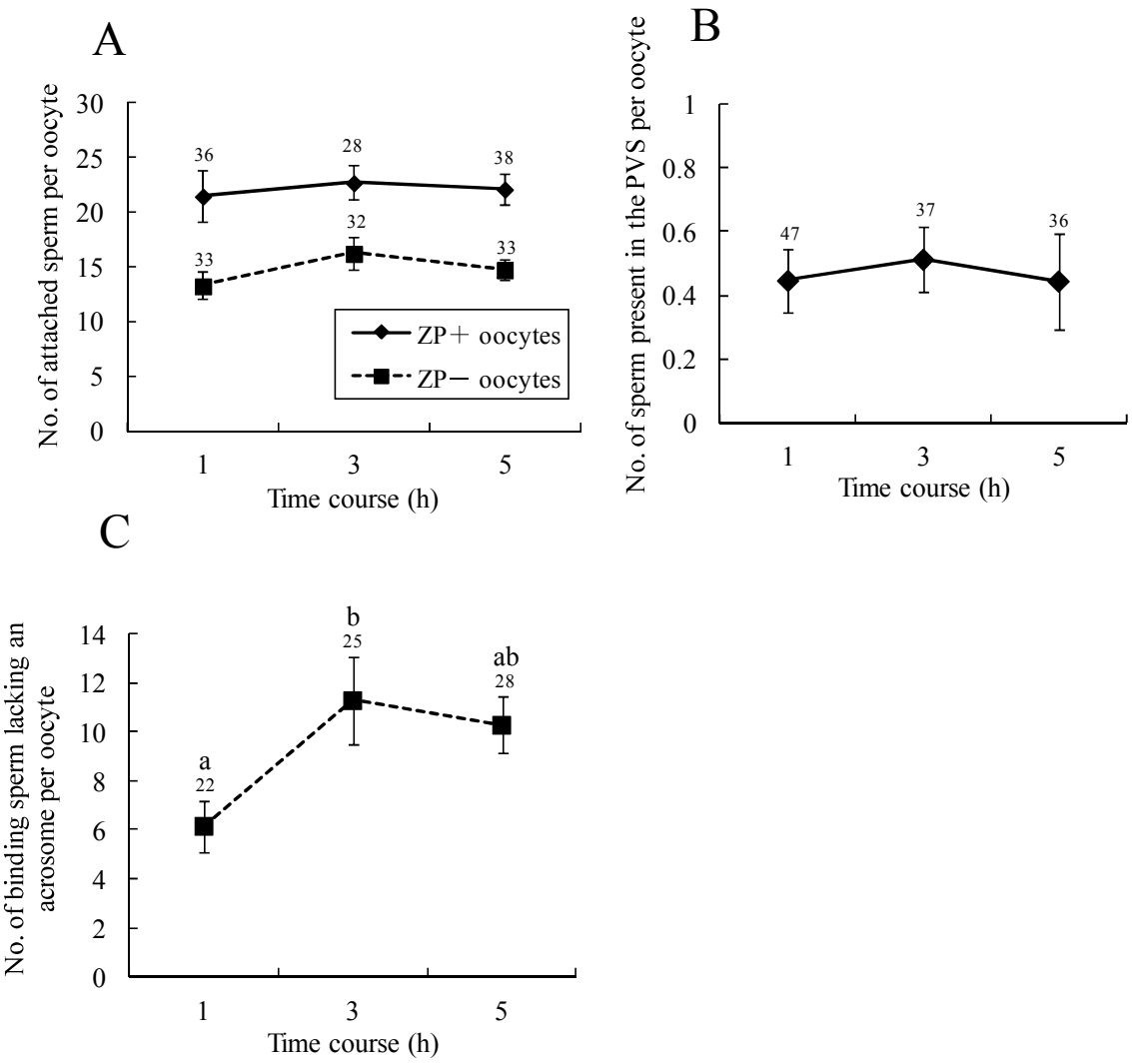

Fig. 2−1 Average numbers of sperm binding to the ZP in ZP+ oocytes and the oolemma in ZP− oocytes (A). Average numbers of sperm present in the PVS of ZP+ oocytes (B). Average numbers of sperm lacking an acrosome that became bound to ZP− oocytes fixed at 1, 3, and 5 h after the initiation of co-incubation of gametes (C). ANOVA demonstrated significant differences in the numbers of binding sperm between ZP+ and ZP− oocytes (See Table 2−1). The number of sperm lacking an acrosome but binding to ZP− oocytes increased significantly as the period from the initiation of co-incubation of gametes to fixation was prolonged (C). Different letters (a, b) indicate significant differences among periods ($P<0.05$). Replicated trials were performed more than three times. Numbers above the bars indicate total numbers of oocytes used in the experimental groups. Means ± SEM are presented.

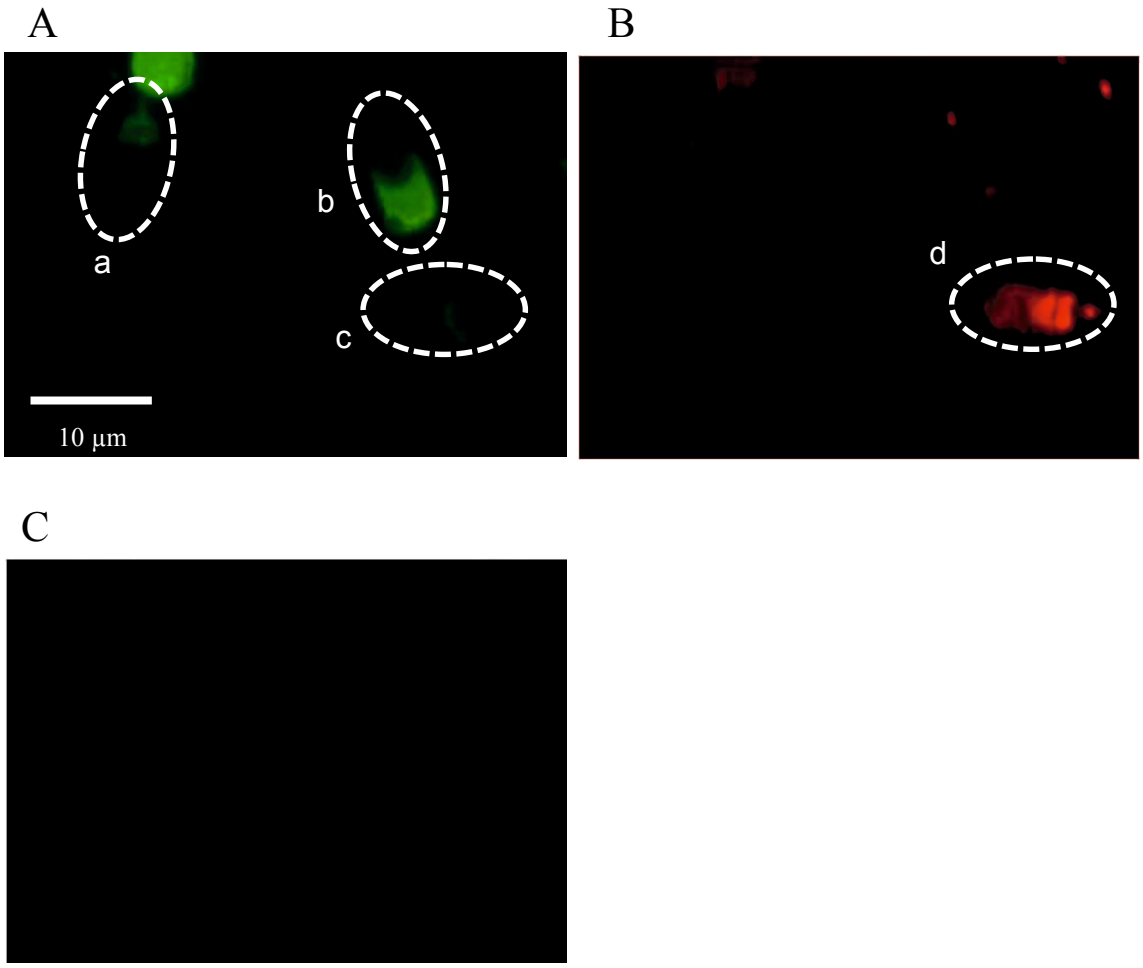

Fig. 2−2 Sperm smear preparations stained with FITC-PNA (A) and with anti-IZUMO antibody (B). Some sperm, which were detected as FITC-PNA-negative, showed spontaneous loss of their acrosomes (a, c). Other sperm, which were detected as FITC-PNA-positive, had intact acrosomes (b). Some of the FITC-PNA-negative sperm were immunopositive for IZUMO (d). When immunostaining was performed without the first antibody, no signals were detected in any (either acrosome-lost or -intact) sperm (C).

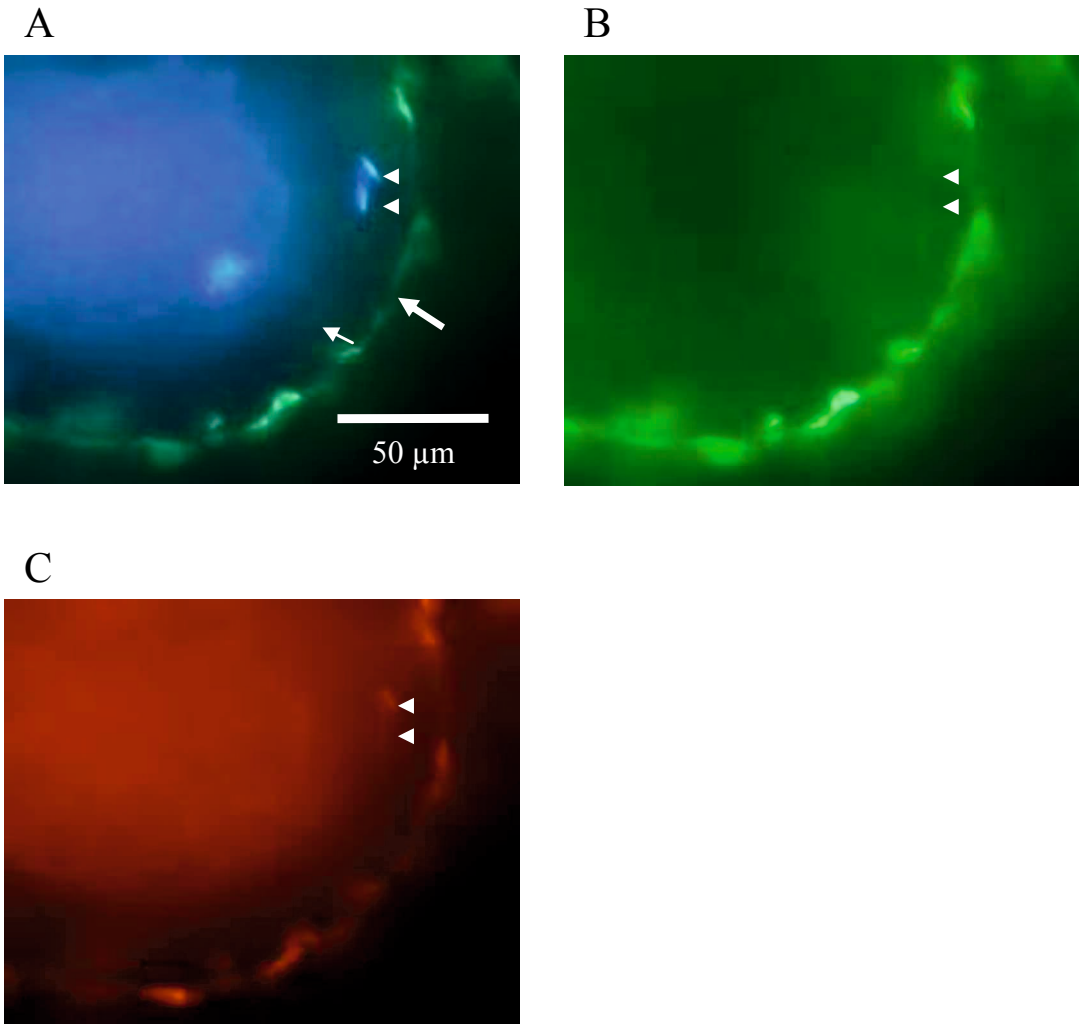

Figure 2−3 Sperm (arrowhead) present in the PVS (small arrow) of a ZP+ oocyte. The large arrow indicates the ZP. The oocytes were stained with Hoechst-33342 (A), FITC-PNA (B), and anti-IZUMO antibody (C). The sperm in the PVS whose heads were stained with Hoechst-33342 lacked an acrosome (FITC-PNA-negative) and were immunopositive for IZUMO.

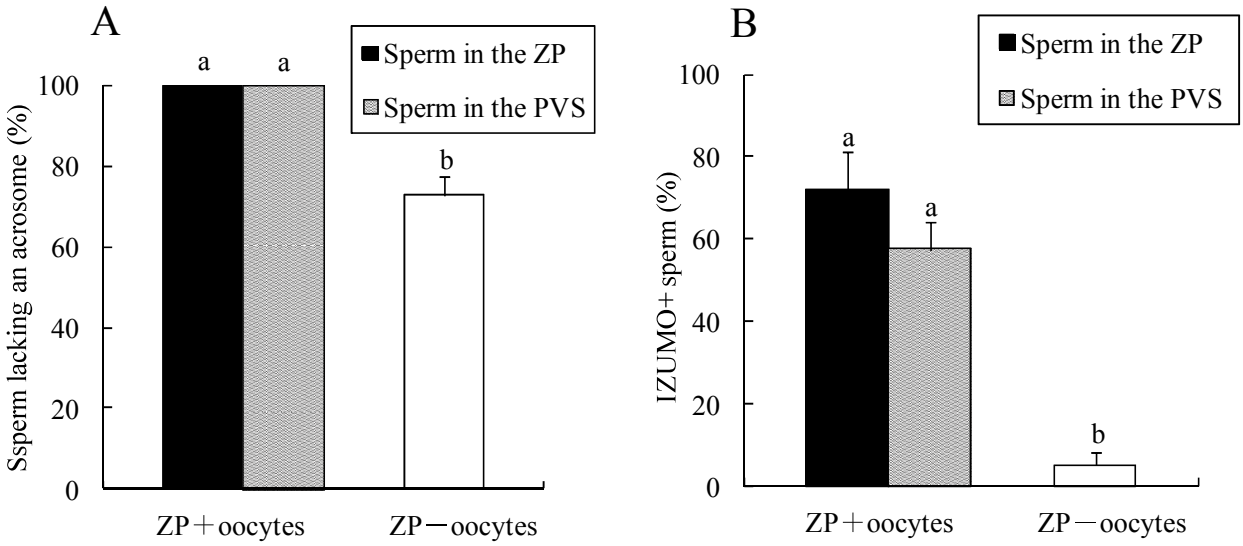

Fig. 2−4 The proportions of sperm lacking an acrosome (A) and IZUMO+ sperm (B) in ZP+ and ZP− oocytes fixed at 3 h after the initiation of co-incubation of gametes. ANOVA demonstrated that a significantly higher proportion of sperm that were passing (in the ZP), or had passed through the ZP (in the PVS) were IZUMO+, in comparison with sperm that had bound to the surface of ZP− oocytes. A: A total of 287 and 433 sperm were analyzed in a total of 60 ZP+ and 62 ZP− oocytes, respectively. B: A total of 287 and 305 sperm were analyzed in the same samples for 60 ZP+ and 62 ZP− oocytes, respectively. Different letters (a, b) indicate significant differences between the two categories of oocytes ($P<0.01$). Experiments were repeated five times. Means ± SEM are presented.

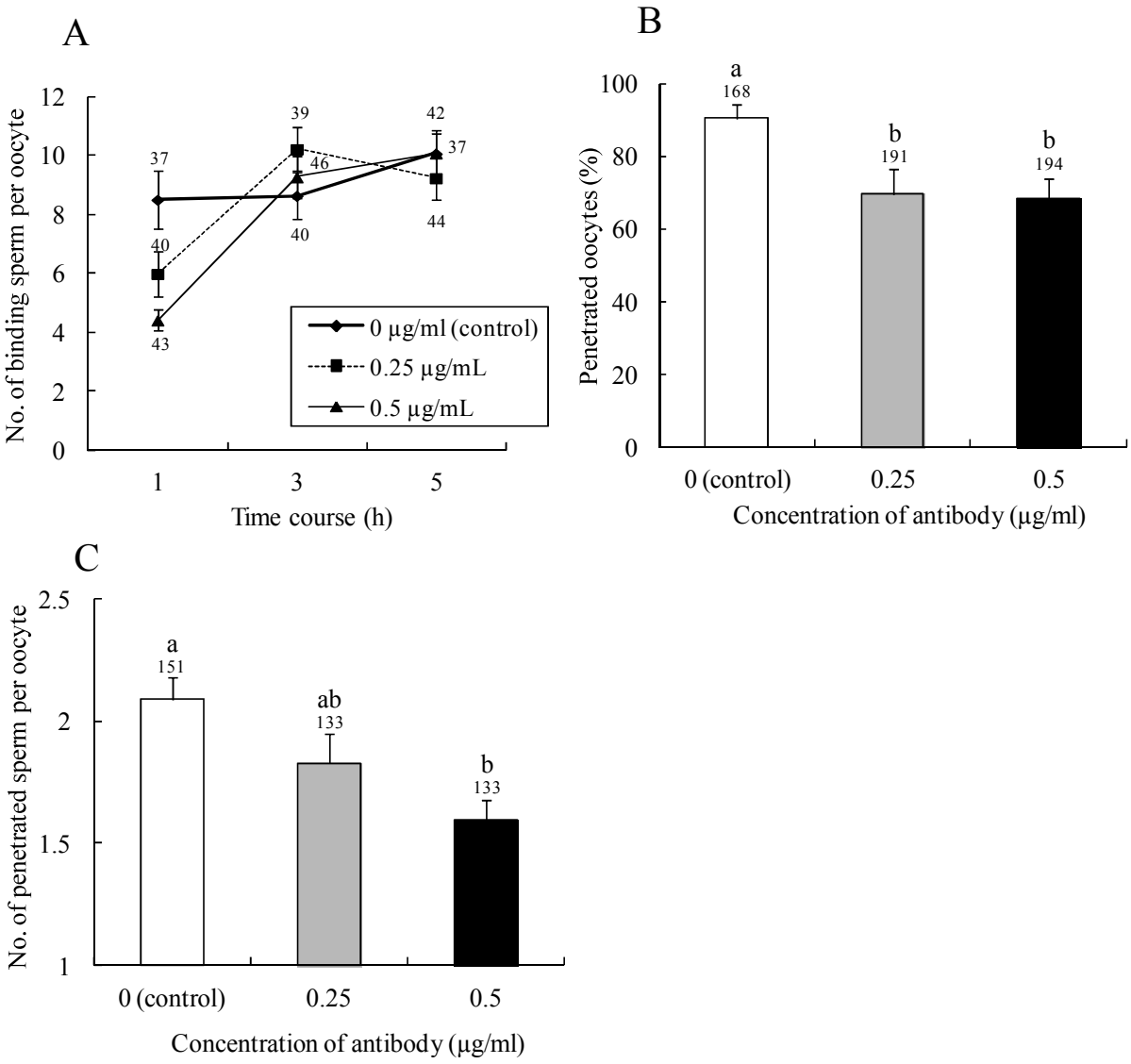

Fig. 2−5 Average numbers of sperm that became bound to the oolemma in ZP− oocytes fixed at 1, 3, and 5 h after the initiation of co-incubation of gametes (A). The proportion of penetrated oocytes (B) and the average number of penetrated sperm per oocyte fixed at 10 h after the initiation of co-incubation of gametes (C). Anti-IZUMO antibody was added to the fertilization medium (0 μg/ml as a control, 0.25, or 0.5 μg/ml). The results of ANOVA are shown in Table 2−2. ANOVA demonstrated no differences in the number of binding sperm among the experimental groups (A). Replicated trials were performed three times. On the other hand, when anti-IZUMO antibody was added to the medium, sperm penetration was significantly inhibited (B, C). Different letters (a, b) indicate significant differences among the experimental groups ($P<0.01$). Replicated trials were repeated six times for each group. Numbers above the bars indicate the total numbers of oocytes used in the experimental groups. Means ± SEM are presented.

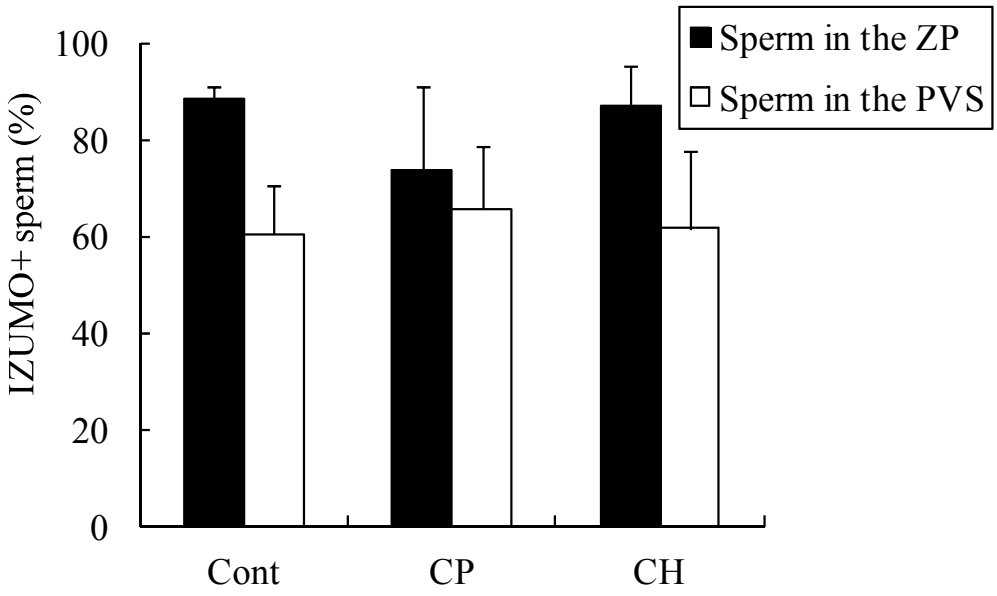

Fig. 2−6 The proportions of IZUMO+ sperm in ZP+ oocytes treated by CP (0.1mg/ml) and CH (1mg/ml) fixed at 3 h after the initiation of co-incubation of gametes. ANOVA demonstrated that there is no significant difference in the sperm that were passing (in the ZP), or had passed through the ZP (in the PVS) among experimental groups. A total of 92, 80, and 62 sperm were analyzed in a total of 36 ZP+ oocytes, 40 ZP+ oocytes treated by CH, and 30 ZP+ oocytes treated by CH, respectively. Experiments were repeated three times for each group. Means ± SEM are presented.

Table 2-1. ANOVA comparing the numbers of sperm bound to oocytes according to presence of the zona pellucida (ZP) and period from the initiation of co-incubation of gametes to fixation.

Source	Number of sperm binding to oocytes		
	df	Mean square	F value
Presence of ZP	1	2724.248	33.30*
Period from the initiation of co-incubation of gametes	2	71.588	0.88

ZP: intact (ZP+) or removed (ZP−). Period from the initiation of co-incubation of gametes to fixation: 1, 3, and 5 h. *df:* degree of freedom

* *P <0.01*

Table 2-2. ANOVA comparing the numbers of sperm bound to oocytes according to the concentration of anti-IZUMO antibody added to Pig-FM and the period from the initiation of co-incubation of gametes to fixation.

Source	Number of sperm binding to oocytes		
	df	Mean square	F value
Concentration of antibody	2	37.583	1.65
Period from the initiation of co-incubation of gametes	2	464.178	20.40*

Concentration: 0, 0.25, or 0.5 μg/ml. Period from the initiation of co-incubation of gametes to fixation: 1, 3, and 5 h. *df:* degree of freedom

* *P <0.01*

OVERALL DISCUSSION AND FUTURE DIRECTIONS

In ARTICLE 1, I investigated the functions of the ZP and oolemma to prevent polyspermy. The ZP is expected to have effective roles to prevent polyspermy *in vitro*. However, these results suggest that the ZP and oolemma of *in vitro* matured oocyte are not able to prevent extra sperm penetration into ooplasm during IVF. Coy *et al*. (2010) investigated the resistance of the ZP to proteolytic digestion before and after fertilization in mammalian, human, cow, and pigs. While in mice and rats, ZP resistance was significantly higher after fertilization or artificial activation, there was no clear pattern in humans and this increase of resistance did not take place in cow or pigs. The ZP resistance to proteolysis after fertilization varies widely among species. However, after treatment of oviductal fluid, the ZP resistance to proteolytic digestion became high in these animals. The function of oviduct may relate to the ZP function, resulting in preventing polyspermy. On the other hand, in human oocytes, Mio *et al*. (2012) observed effective mechanism of polyspermy block which is different from previous mechanisms; polyspermy block as the zona reaction and the oocyte membrane block to sperm penetration during IVF in mice. This mechanism takes place in the ZP. The ZP has further unknown functions involved in sperm penetration. The results in ARTICLE1

suggest that the existence of ZP accelerates sperm penetration in pigs. This result is considered to indicate one of the unknown functions of ZP excepting for zona reaction, therefore, I investigated the details of ZP function for sperm in ARTICLE 2.

It is known that the ZP is important to induce AR. In ARTICLE 2, I showed that all of the sperm which were passing or had passed through the ZP lost their acrosome, and they fused to oolemma with high efficiency. Inducing AR is thought to be one of the important functions of ZP to accelerate successful sperm penetration. However, sperm penetration was significantly less in ZP− oocytes than that in ZP+ oocytes although many sperm without acrosome spontaneously bound to oolemma in ZP− oocytes. The sperm that were induced AR (lost their acrosome) after passing through the ZP have obviously higher ability to fuse with oolemma than that of sperm without acrosome spontaneously. The ZP was possible to have function(s) to accelerate sperm-oolemma fusion.

In recent years, sperm-oolemma fusion factors have been well studied. IZUMO is critical factor for sperm-egg fusion on sperm, and the results in my study suggest that IZUMO is also important factor for sperm penetration in porcine species and the functional exposure of IZUMO is induced by the ZP. Passage of the ZP induces AR and functional exposure of IZUMO resulting in acceleration of sperm penetration into the

ooplasm in frozen-thawed boar epididymal spermatozoa. This mechanism may lead the result that sperm penetration was higher in ZP+ oocytes than that in ZP− oocytes (results of ARTICLE 1). The results also suggest that sperm suffering spontaneous loss of the acrosome often fail to expose IZUMO. Furthermore, in ARTICLE 2, I suggest that IZUMO may not be synthesized after contact to the ZP. IZUMO may be considered to be exposed after some kind of modifications, such as redistribution or post-translational modification during IVF. I expect that to identify the factor(s) inducing the functional exposure of IZUMO in the ZP is possible to apply the improvement of efficiency of sperm penetration and generating embryos originated in precious individuals, whose sperm has low fertilization ability. In human, Clark *et al*. (2013) suggests relationship between infertility and anti-IZUMO antibody in sera of infertile women and men. I also expect that the factor(s) is able to be contributed to overcome infertility in human.

Subzonal injection of spermatozoa (SUZI) was one of the micromanipulation techniques to overcome infertility by injecting sperm in the PVS. It is efficient in overcoming a certain male factor infertility and unexplained *in vitro* fertilization failures. Today, SUZI has been progressively replaced by intracytoplasmic sperm injection (ICSI) (Patrat *et al*. 1999). ICSI is an assisted-fertilization technique which involved

injection of a single sperm through the ZP directly into the ooplasm (Palermo *et al*. 1992). When the results of SUZI and ICSI were compared, fertilization rate was significantly higher in patients performing ICSI than that in patients performing SUZI (Abdalla *et al*. 1995, Imthurn *et al*. 1995). However, in case of performing ICSI, it is possible that braking of ooplasm or leaking out of ooplasm may occur after injection. This is considered to be under unphysiological situation during fertilization. Establishment of method to induce functional exposure of IZUMO efficiently may enable the acceleration of sperm penetration in case of performing SUZI or other assisted-fertilization techniques bypassing the ZP as one of the choices achieving a successful fertilization safely.

Berger *et al.* (1989) reported that some components isolated from the ZP induced the AR. Furthermore, CD81, one of the tetraspanin localizing in the ZP, involves in the sperm-oolemma fusion event and inducing the AR in mice (discussed later) (Tanigawa *et al.* 2008). On the other hand, the porcine ZP is composed of three glycoproteins: ZPA/ZP2, ZPB/ZP4, and ZPC/ZP3 (Goudet *et al.* 2008), and it has been reported that ZPB/ZP4-ZPC/ZP3 complexes show specificity for porcine spermatozoa (Yurewicz *et al.* 1998). These components and/or structure of the ZP may induce not only the AR but also functional exposure of IZUMO. Further studies to evaluate the factor(s) in the ZP

that affect the functional exposure of IZUMO are needed.

I have shown that removal of the ZP inhibits sperm penetration (ARTICLE 1) and the ZP accelerates AR and functional exposure of IZUMO, important factor for sperm-oolemma fusion (ARTICLE 2). However, removal of the ZP may have other demerit(s) in sperm penetration excepting failure of AR and functional exposure of IZUMO. After removal of ZP, the PVS exposed to the fertilization medium. There may be some important factor(s) or mechanism(s) on the oolemma and/or in the PVS, which also participate in sperm penetration for completion of fertilization. When the ZP is removed, these factors or mechanisms may be lost upon direct exposure of the PVS and/or oolemma to the IVF medium. In bovine oocytes, it has been reported that fibronectin is present in the PVS, which is one of the factors related to sperm-oolemma binding. However, when the ZP is treated with protease, this factor may be removed from the periphery of the oocyte (Thys *et al*. 2009).

Fibronectin is also considered to have important roles for sperm penetration in pigs, as one of the factors presenting in oocytes and involving in sperm penetration. Mattioli *et al.* (1998) suggest that fibronectin, component of extracellular matrix in the cumulus mass that surrounds oocyte, stimulated AR in pigs. Fibronectin is also well studied in human. Henkel *et al.* (1996) suggested that fibronectin, as an adhesion molecule, is

intimately involved in the sperm-oolemma interaction. Fusi *et al*. (1992) also reported that sperm surface fibronectin is expressed following capacitation, and anti-fibronectin antibody reduces sperm penetration with hamster oocytes in human. Bronson *et al.* (1995) suggest that addition of echistatin, a disintegrin known to block the binding of fibronectin, reduce sperm adherence to the oolemma significantly at micromolar concentrations of echistatin in human. Fibronectin is one of the important factors involved in sperm-oolemma interactions; however it contains unknown function(s). Further investigation will be necessary.

In addition, CD9 has also been reported to be an important factor for sperm-oolemma fusion in mouse, bovine, and porcine oocytes (Li *et al*. 2004, Miyado *et al*. 2008, Zhou *et al*. 2009). Another research has indicated that mouse oocytes incubated with pronase to remove the ZP lose all their CD9 from the oolemma (Komorowski *et al*. 2003). Ohnami *et al.* (2012) suggested that CD9 localized not only at the oolemma, but also at the PVS in mice. Removal of the ZP, resulting in the lost of PVS, may indicate lost of their CD9. They also reported that CD81 was observed in ZP. Tetraspanin CD81 is closely homologous in amino acid sequence with CD9 and CD81 and has also been reported to be involved in sperm-oolemma fusion event. In mice, when CD81-deficient oocytes are subjected to IVF with normal sperm, the

sperm-oolemma fusion rate is reduced, and some of the sperm penetrating into the PVS fail to undergo AR (Tanigawa *et al.* 2008). Removal of the ZP also indicates the lost of CD81. Further studies focusing on the oocytes factor(s) relating to fertilization will be needed.

In the present study, I elucidated one of the functions of ZP during IVF, accelerating functional exposure of IZUMO resulting in successful sperm penetration. The ZP supports success of normal fertilization not only by being barrier to extra sperm penetration, but also by getting condition of sperm for fusing with oolemma ready. Researches in pigs as an experimental model are expected to be applied to research in human. I expect that this investigation in pigs presents some possibilities for contributing the improvement of porcine reproduction system, resulting in the improvement of reproductive biology, and medical and pharmaceutical industry including human.

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