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—Original Article—

Evaluation of Zona Pellucida Function for Sperm Penetration During *In Vitro* Fertilization in Pigs

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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, we investigated the effects of the ZP on sperm penetration during *in vitro* fertilization (IVF). We 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 evident difference in the parameters between the two groups. Secondly, we 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, we 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, we 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 in our present porcine IVF system. However, it appears that ZP removal is one of the possibilities for reducing polyspermic penetration *in vitro* in pigs.
Key words: Fertilization, Oocyte, Pig, Polyspermy, Zona pellucida

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In human oocytes, malfunction of the zona pellucida (ZP) [1] and anti-zonal antibodies [2–4] have been reported to be a cause of infertility and failure of *in vitro* fertilization (IVF), and abnormality of the ZP is also one of the causes of polyspermic penetration [5]. It is expected that spermatozoa can easily penetrate into an oocyte after removal of the ZP [6]. 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 [7, 8], 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 [8, 9], sperm binding [10] and prevention of polyspermy [5, 11, 12]. Removal of the ZP may have unexpected influences on these functions.

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 and research on human regenerative medicine. The application of porcine embryonic stem cells is also expected for efficient production of normal embryos. In porcine oocytes, however, polyspermy occurs with high frequency and is considered to be an obstacle for efficient *in vitro* production of normal embryos [13, 14]. In mammalian oocytes, the most accepted mechanism for prevention of polyspermy is modification of the ZP through release of cortical granules (zona reaction) [5, 15]. After these biochemical and structural changes, the ZP loses its ability to bind and be penetrated by sperm [16–18]. It is also known that the porcine ZP does not prevent polyspermy, especially in *in vitro*-matured porcine oocytes [14]; however, the function of the ZP in this species remains insufficiently understood.

In the present study, we examined the roles of the porcine ZP in sperm penetration and polyspermy prevention. First, we evaluated the effects of pronase treatment of the ZP on sperm penetration. Pronase is a protease that has been used widely to dissolve/remove the ZP. Second, we investigated the function of the ZP in sperm penetration using porcine oocytes from which the ZP had been removed. Third, to elucidate whether the ZP and/or oolemma functions to prevent polyspermy, we evaluated the penetration parameters of oocytes with or without the ZP. Finally, we focused on the function of the oolemma in prevention of polyspermy.

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Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Collection and IVM of porcine oocytes were carried out as reported previously [19]. 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, St Louis, MO, USA) supplemented with 5% (v/v) fetal bovine serum (Gibco, Life Technologies, 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 4-well dishes (Nunclon Multidishes; Thermo Fisher Scientific, Waltham, NA, USA). The medium employed was modified North Carolina State University (NCSU)-37 solution [20] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 mM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (Puberogen 500 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

We 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 PBS (Nissui Pharmaceutical, Tokyo, Japan) for 20−30 sec [21]. 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 [22]. First, we stabbed the ZP with a glass needle and formed a slit in it. Next, we 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 [19]. In brief, epididymides were isolated from Landrace boars, and epididymal spermatozoa were collected from them and frozen [23]. Spermatozoa were thawed and preincubated for 15 min in Medium 199 with Earl's salts (Gibco) adjusted to pH 7.8 [24]. Oocytes were transferred to fertilization medium for porcine oocytes (Pig-FM) [25], in which the caffeine concentration was modified to 5 mM [26]. 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. In the present study, the initiation of IVF (introduction of sperm to IVF medium containing oocytes) was termed "insemination." 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) [19]. 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: We evaluated the effects of pronase treatment of oocytes on sperm penetration. We prepared ZP-free oocytes as follows. In the first group, mZP− oocytes were incubated for 1 h in IVM medium**.** In the second group, we supplied pZP− 1 h oocytes. Finally, in the third group, we 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 insemination, oocytes in all the groups were fixed, and their sperm penetration parameters were evaluated.

Experiment 2) Effects of ZP on sperm penetration: We 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 insemination, 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. We evaluated sperm penetration at 1, 2, 3, 4, 5 and 10 h after insemination. 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, we 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, we 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. We fixed the oocytes at 3, 5 and 10 h after insemination and then stained

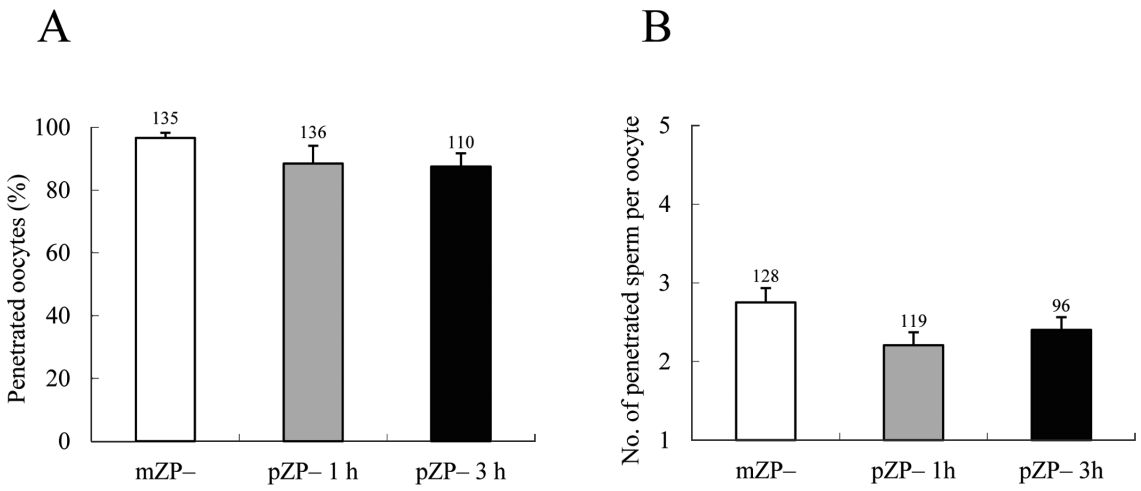

Fig. 1. The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) in each of the treatment groups fixed at 10 h after insemination (initiation of *in vitro* fertilization). mZP− oocytes were denuded of the zona pellucida without pronase treatment. The other two groups, pZP− 1 h and pZP− 3 h oocytes, were treated with pronase to remove the zona pellucida 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.

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, Cary, NC, USA). Percentage data were arcsine-transformed before the analysis.

Results

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

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. 1A and 1B, respectively. Only oocytes penetrated by sperm were used for calculation of the average number of penetrated sperm per oocyte. After ANOVA, we 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, we used pZP− 1 h oocytes as zona-free oocytes (hereafter termed ZP− oocytes).

Experiment 2: Effects of ZP on sperm penetration

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. 2A and 2B. The results of ANOVA are shown in Table 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, we therefore used these sperm with the expectation of obtaining clearer results.

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

The combined effects of the ZP present during IVF and the period from insemination to fixation are shown in Fig. 3A and 3B. The results of ANOVA are shown in Table 2. Significant differences were evident for sperm penetration parameters in both ZP+/− groups and as well as the period from insemination. 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 insemination to fixation.

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

The combined effects of the duration of gamete co-incubation (3 and 5 h) and period from insemination to fixation (3, 5 and 10 h) are shown in Fig. 4. The results of ANOVA are shown in Table 3. Significant differences were detected in both the duration of gamete co-incubation and period from insemination. 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 insemination to fixation was prolonged to 10 h.

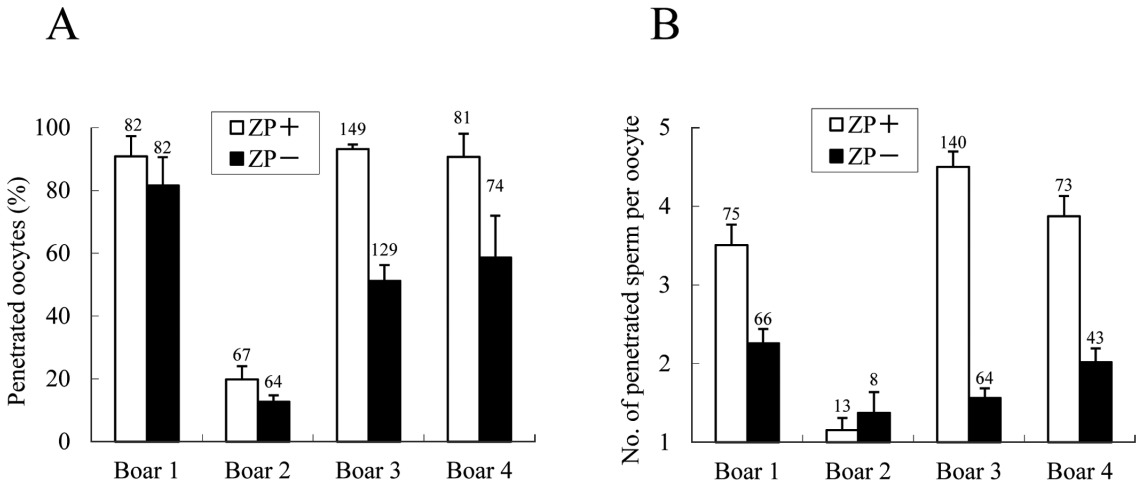

Fig. 2. The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) in ZP+ and the ZP− oocytes fixed at 10 h after insemination (initiation of *in vitro* fertilization). Frozen-thawed epididymal spermatozoa from 4 different boars were used (Boars 1−4). The results of ANOVA are shown in Table 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.

Table 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	458.33	124.42[a]
Boar	3	1.05	19.79[a]	3	31.428	8.53[a]
Interaction between ZP and Boar	3	0.119	2.24	3	27.83	7.55 [a]

ZP: intact (ZP+) or removed (ZP−). Boar: 4 boars. *df*: degree of freedom. [a] P<0.01.

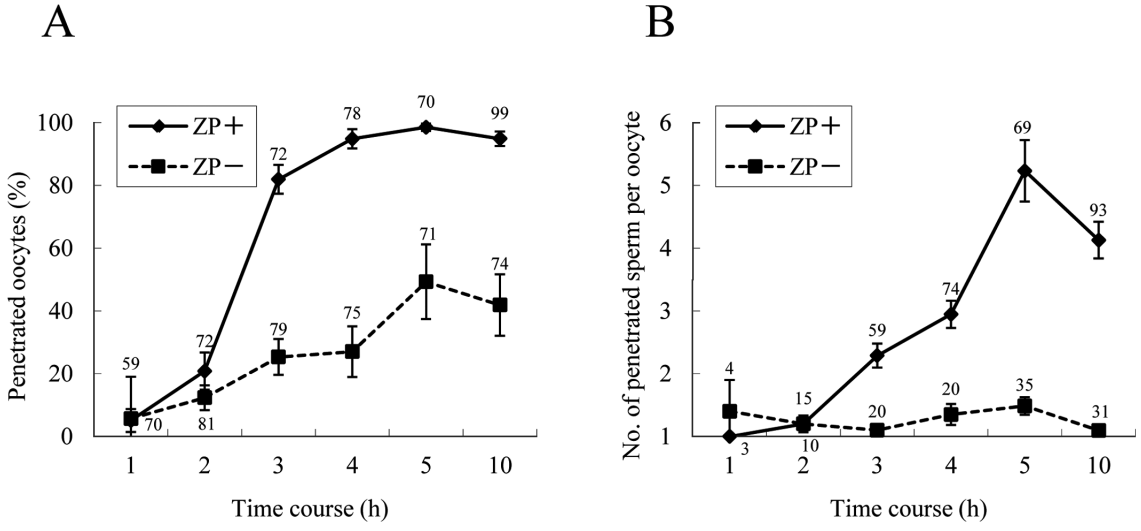

Fig. 3. The proportion of penetrated oocytes (A) and the average number of penetrated sperm per oocyte (B) for ZP+ and ZP− oocytes at 1, 2, 3, 4, 5 and 10 h after insemination (initiation of *in vitro* fertilization). We used frozen-thawed epididymal spermatozoa from one lot (Boar 3 in Fig. 2), for which 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 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.

Table 2. ANOVA of sperm penetration parameters according to presence of the zona pellucida (ZP) and period from insemination 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 insemination	5	1.741	34.12[a]	5	64.144	11.85[a]
Interaction between ZP and insemination	5	0.745	14.60[a]	5	26.452	4.89[a]

ZP: intact (ZP+) or removed (ZP−), Period from insemination to fixation: 1, 2, 3, 4, 5 and 10 h. *df:* degree of freedom. [a] P<0.01.

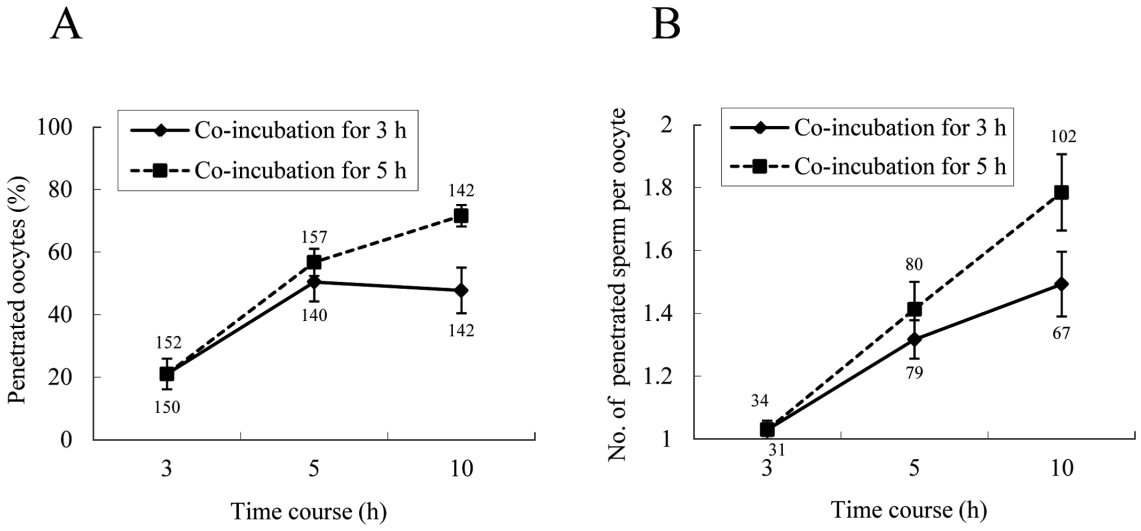

Fig. 4. The proportion of penetrated ZP− oocytes (A) and the average number of penetrated sperm per oocyte (B) in the control (co-incubation for 3 h) and prolonged co-incubation groups (co-incubation for 5 h) at 3, 5 and 10 h after insemination (initiation of *in vitro* fertilization). We used the same frozen-thawed epididymal spermatozoa (Boar 3 in Fig. 2). The results of ANOVA are shown in Table 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 3. ANOVA of sperm penetration parameters into ZP-free oocytes according to duration of gamete co-incubation and period from insemination 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 insemination	2	0.549	28.68[b]	2	10.869	15.67[b]
Interaction between co-incubation and insemination	2	0.06	3.14	2	1.336	1.93

Duration of gamete co-incubation: 3 and 5 h. Period from insemination to fixation: 3, 5 and 10 h. *df:* degree of freedom. [a] P<0.05; [b] P<0.01.

Discussion

Recently, an *in vitro* production system for porcine embryos has been developed [27–29]. However, polyspermy is considered to be a very troublesome obstacle to efficient production of normal porcine embryos because although polyspermic oocytes can develop to blastocysts, their ploidy becomes abnormal [30, 31]. 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 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 [5, 32–34]. Kim *et al.* [33] reported that addition of 1.0% oviductal fluid to the fertilization medium increased monospermy. Coy *et al.* [32] 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 *et al.* [34] demonstrated that 2.5 h co-culture of sperm and oviduct cells reduces polyspermy. However, the mechanism responsible for polyspermy is still not well understood, and efforts to clarify it have been limited. As mentioned above, the zona reaction is important for prevention of polyspermy in mammalian oocytes. Therefore, we evaluated the roles of the ZP during IVF to help clarify the mechanism of polyspermy in pigs.

To understand the function of the ZP in sperm penetration and blocking of multiple sperm entry, we compared sperm penetration in both ZP+ and ZP− oocytes. Usually, ZP− oocytes can be obtained easily by treatment with pronase (protease) (for example, in porcine [8, 35, 36], bovine [37, 38] and mouse [6, 39] oocytes). However, we hypothesized that this enzyme treatment might exert some negative effects on sperm penetration (or prevention of polyspermy) in porcine oocytes. Initially, therefore, we evaluated the effects of pronase treatment of oocytes on sperm penetration in Experiment 1. Using mouse oocytes, Yamagata *et al.* [22] succeeded in removing the ZP using a micromanipulator. Thus, in the present study, we also removed the ZP mechanically using a micromanipulator without pronase treatment 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, we 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. Pronase is a protease separated from the extracellular fluid of *Streptomyces griseus* [40]. Wolf *et al.* [6] 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.* [39] 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 [23, 41]. Furthermore, from these results, we suggest that when the ZP is not present, sperm penetration into oocytes cannot be accelerated. The acrosome reaction (AR) plays very important roles in sperm penetration. Acrosome-intact or partially acrosome-reacted sperm can bind to the ZP [14], and thereafter the AR is induced by the ZP [8, 9]. 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 [42]. On the other hand, in the present study, a certain proportion of ZP− oocytes was also penetrated. Wu *et al.* [8] 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") [41, 43] and are considered to lose their acrosome spontaneously during incubation in fertilization medium. Therefore, in our 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 insemination. In Experiment 3, therefore, to clarify whether polyspermy was prevented by the ZP and/or oolemma, we evaluated sperm penetration parameters with time after insemination. The results clearly demonstrated that sperm penetration increased significantly with time after insemination. 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 [15, 44]. In porcine *in vivo*-matured oocytes, the zona reaction is induced during fertilization [35]. On the other hand, in *in vitro*-matured porcine oocytes, some researchers have reported that the zona reaction is incomplete or delayed [45–47]. Hatanaka *et al.* [36] 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 (after release of cortical granules) differ between *in vivo*- and *in vitro*-matured porcine oocytes [46]. Furthermore, the structure of the ZP and its resistance to pronase digestion may similarly differ *in vivo* and *in vitro* [35, 46]. 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. We 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 (membrane block) after the first sperm penetration. Therefore, in Experiment 4, we prolonged gamete co-incubation from 3 h (standard duration in our 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) [48]. 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 [49]. Among mammalian species, the mechanism of membrane block has been examined only in mice [49, 50]; however, in porcine oocytes,

no studies have investigated this issue. In the present 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 [49]. McAvey *et al.* [51] 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 insemination. 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 [52–54]. Elevation of intracellular calcium levels (corresponding to oocyte activation) is important for the establishment of membrane block in mouse oocytes [51]. We are not sure if there is a similar mechanism for membrane block in porcine oocytes because there has been no report about this phenomenon. Our 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.

Another important factor(s) or mechanism(s) on the oolemma and/or in the perivitelline space may participate in sperm penetration for completion of fertilization. When the ZP is removed, this factor or mechanism may be lost upon direct exposure of the perivitelline space and/or oolemma to the IVF medium. For example, in bovine oocytes, it has been reported that fibronectin is present in the perivitelline space and that this is a factor related to sperm-oolemma binding. However, when the ZP is treated with protease, this factor may be removed from the periphery of the oocyte [55]. CD9 has also been reported to be an important factor for sperm-oolemma fusion in mouse, bovine and porcine oocytes [56−58]. Other research has indicated that mouse oocytes incubated with pronase to remove the ZP lose all their CD9 from the oolemma [59]. Our present findings support this possibility. Further studies will need to focus on the reasons for our present results.

In conclusion, the ZP and oolemma are not competent factors for prevention of polyspermy, at least in our present porcine IVF system. However, it appears that ZP removal is one of the possibilities to reduce polyspermic penetration *in vitro* in pigs.

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