Egg Envelope Glycoproteins ZP1 and ZP3 Mediate Sperm-Egg Interaction in the Japanese Quail

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Fertilization is indispensable for zygotic formation leading to the birth of animals and the species-specific sperm-egg binding thought to be the initial step in this important process. In birds, the oocyte, which encounters the spermatozoa at the time of fertilization, is enclosed in a perivitelline membrane (pvm) constructed of several zona pellucida glycoproteins (ZP proteins: ZP1, ZP2, ZP3, ZP4 and ZPD). The aim of this study was to determine the ZP protein in the pvm responsible for sperm-pvm binding in Japanese quail. We tested the effects of anti-ZP protein antibodies on *in vitro* sperm perforation in the pvm. The results showed that the anti-ZP1 and ZP3 antibody significantly blocked hole formation by sperm, whereas anti-ZP2, ZP4 and ZPD as well as normal rabbit serum had no such effect. When the sperm acrosome reaction was inhibited in the presence of pertussis toxin, sperm-pvm binding was observed. This sperm-pvm binding was significantly prevented when the purified ZP1 or ZP3 was included in the reaction mixture. Moreover, both digoxigenin-labeled ZP1 and ZP3 were found to interact with the sperm head by immunocytochemical observation. Our results indicate that sperm binding to the pvm is, at least in part, mediated by the interaction of ZP1 and ZP3 with the sperm head during fertilization in Japanese quail.

Key words: fertilization, Japanese quail, perivitelline membrane, sperm, sperm-egg binding

Introduction

Fertilization comprises sequential steps including sperm-egg binding, induction of acrosomal reaction (AR), sperm penetration through the oocyte, and sperm-egg fusion. It is indispensable for zygotic formation leading to embryonic development. Sperm-egg binding is the initial step in fertilization and an extracellular coat surrounding the oocyte, referred to as a zona pellucida (ZP) in mammals and a perivitelline membrane (pvm) in birds, plays an important role in this step. These egg extracellular coats are mainly constituted of glycoproteins belonging to different subclasses of the ZP gene family (Spargo and Hope, 2003; Conner *et al*., 2005; Smith *et al*., 2005). In mammals, this matrix is composed of three or four glycoproteins (*i.e.* ZP1, ZP2, ZP3 and ZP4), (Lefievre *et al*., 2004; Hoodbhoy *et al*., 2005; Litscher and Wassarman, 2007; Ganguly *et al*., 2008; Izquierdo-Rico *et al*., 2009). By means of a classical com-

petitive sperm-egg binding assay in which each purified ZP protein was included in the incubation mixture of sperm and egg, it has long been understood that ZP3 is responsible for sperm-egg binding in mice (Florman and Wassarman, 1985). However, recent research using humanized ZP in transgenic mice indicates that human sperm specifically bind to the N-terminal domain of ZP2 (Baibakov *et al*., 2012).

In avian species, pvm is composed of at least five glycoproteins (ZP1, ZP2, ZP3, ZP4, and ZPD) in Japanese quail (Pan *et al*., 2001; Sasanami *et al*., 2003; Sato *et al*., 2009; Kinoshita *et al*., 2010; Serizawa *et al*., 2011) and 6 glycoproteins (ZP1, ZP2, ZP3, ZP4, ZPD, and ZPAX) in chicken (Smith *et al*., 2005). ZP1 and ZP3 have been identified as major constituents in the pvm of both species. Elucidation of the mechanism of sperm-egg interaction in birds is hampered because no available method for *in vitro* insemination exists to date. The oocytes are too large to study by direct observation of the sperm-egg interaction *in vitro* and the ovulated oocytes quickly lose their fertilizability because the chalaza-layer, also referred to as the outer layer of the vitelline membrane secreted from the infundibulum part of the oviduct, overlays the surface of the pvm immediately after ovulation (Wishart, 1997). The sperm no

longer interact with these oocytes thereafter and are embedded in the chalaza-layer (Wishart, 1997). Therefore, researchers incubate the isolated pvm with ejaculated sperm in vitro as an alternative model for *in vitro* fertilization (IVF) (Birkhead *et al.*, 1994; Robertson *et al.*, 1997; Robertson *et al.*, 1998; Kuroki and Mori, 1997; Sasanami *et al.*, 2012). Using this technique, parts of the mechanisms of sperm-egg binding in birds have been identified. In the Japanese quail, we previously demonstrated that acrosin, which localizes on the plasma membrane of the sperm in addition to the acrosomal matrix, supports sperm egg binding (Sasanami *et al.*, 2011). Although acrosin has the ability to bind with the pvm, the binding partner of acrosin in the pvm is not known.

The aim of this study was to determine which component in the pvm is responsible for sperm-pvm binding, the first step of sperm-egg interaction in the Japanese quail.

Materials and Methods

Animals and Tissue Preparation

Male and female Japanese quails, *Coturnix japonica*, 8–20 weeks of age (Motoki Corporation, Tokorozawa, Japan), were maintained individually under a photoperiod of 14 h light: 10 h darkness (light at 0500 h) and provided with water and a commercial diet (Motoki Corporation) *ad libitum*. The females were decapitated and the largest follicle was dissected. The granulosa layer from the largest follicle was isolated as a sheet of granulosa cells sandwiched between the pvm and basal laminae, as described previously (Gilbert *et al.*, 1977). The pvm was isolated according to the procedure described by Sasanami *et al.* (2002). The isolated pvm was used in an *in vitro* assay for sperm–egg interaction and purification of the ZP1 and ZP3 proteins. All experimental procedures for the care and use of animals in this study received approval from the Animal Care Committee, Shizuoka University (approval number, 28–13).

ZP1 and ZP3 Purification

The isolated pvm was dissolved in 1% SDS (w/v) buffered at pH 6.8 with 70 mM Tris-HCl overnight at room temperature. After centrifugation at 10 000 *g* for 10 min, the supernatants were served as pvm lysates. The pvm lysate was separated by one-dimensional SDS–PAGE, performed as described by Laemmli (1970), under nonreducing conditions on 12% (w/v) polyacrylamide separating gel. The samples were applied to 5% (w/v) stacking gel, without a comb, for lane casting. After electrophoresis, the gel was stained with Copper Stain (Bio-Rad Laboratories), and the 97 kDa ZP1 and 35 kDa ZP3 bands were excised. The proteins were eluted by incubating the gel slices with 0.1% (w/v) SDS buffered at pH 9.0 with 100 mM Tris–HCl overnight at 4℃ with vigorous shaking. The eluent was then extensively dialyzed against water, lyophilized, and dissolved in PBS (-). The protein concentration of the sample was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). The purity of the ZP1 and ZP3 was confirmed by silver staining after separation of the protein by SDS–PAGE.

Semen Collection and Preparation

Ejaculated sperm was obtained from male quail before mating using the procedure described by Kuroki and Mori (1997). Semen obtained from two to three males was suspended in Hanks' balanced salt solution (HBSS) containing 1.25 mM of $CaCl_2$ and 1.8 mM of $MgSO_4$. The concentrations of sperm were measured with a hemocytometer. In all the experiments, sperm were incubated at 39℃.

In Vitro Assay for Sperm-egg Interaction

Pvm cut into approximately 5 mm in diameter was incubated with 0.5 m*l* sperm suspension at 1×10^7 sperm/m*l* in HBSS at 39℃ for 30 min. After incubation, reaction was terminated by placing the tube on ice and the pvm was washed three times with ice-cold PBS, transferred onto a glass slide and stained with Schiff's reagent after fixation with 3.7% (v/v) formaldehyde in PBS. The holes that formed on the pvm were photographed in a light microscope (BX 51, Olympus Optics, Tokyo, Japan), and the number of holes was calculated using Image J software (NIH). At least five areas were randomly selected for enumeration of the perforations.

To observe the effect of anti-ZPs antibodies, the pvm was added into a tube containing either anti-ZP1 raised against the synthetic N-terminal peptide of ZP1 (Ohtsuki *et al.*, 2004), anti-ZP2 raised against bacterially expressed ZP2 (Kinoshita *et al.*, 2010), anti-ZP3 raised against purified ZP3 from the pvm (Sasanami *et al.*, 2002), anti-ZP4 raised against bacterially expressed ZP4 (Serizawa *et al.*, 2011), anti-ZPD raised against bacterially expressed ZPD (Sato *et al.*, 2009) or normal rabbit serum diluted 1:200 with HBSS, and sperm suspension was then added at 1×10^7 sperm/m*l*.

We previously demonstrated that sperm binding to the pvm could be evaluated by inhibiting sperm AR in the presence of pertussis toxin (PTX, Calbiochem, La Jolla, CA, USA) (Sasanami *et al.*, 2011), therefore 2 μg/m*l* PTX was added to the reaction mixture during the sperm-pvm incubation to analyze the sperm-pvm binding. For the analysis of the effects of the purified ZP1 and ZP3 on the sperm-pvm binding, ejaculated sperm stained with Hoechst 33342 (1 μg/m*l*) at 1×10^7 sperm/m*l* in HBSS was mixed with equal amounts of the purified ZP1 or ZP3 diluted in HBSS in the presence of PTX and incubated as described above. BSA was added as a control protein. The pvm was gently washed three times with PBS, transferred onto a glass slide, and fixed by fixation with 3.7% (v/v) formaldehyde in PBS. The numbers of sperm bound on the pvm were counted under a fluorescence microscope equipped with an interference contrast apparatus with a 40× objective (BX 51, Olympus Optics, Tokyo, Japan).

Immunofluorescence Microscopy

Digoxigenin (DIG)-labeled ZP1 and ZP3 were prepared by a DIG Protein Labeling Kit (Roche) as described previously (Kinoshita *et al.*, 2008). Ejaculated sperm were diluted to 1 $\times 10^7$/m*l* and fixed in 3.7% (v/v) formaldehyde in PBS at room temperature for 10 min. The fixed sperm were smeared on poly-L-lysine coated microscope slides. After air drying, the slides were washed with PBS for 5 min, and the sperm incubated with PBS containing 1% BSA and 2% normal goat serum for 1 h for blocking. The sperm were then incubated

with DIG-labeled ZP1 or ZP3 (50 μg/ml) for 2 h at 4℃. After washing 3 times with PBS for 5 min, they were incubated with FITC-conjugated goat anti-DIG IgG (1: 200, Roche) for 1 h at 4℃. After washing with PBS they were embedded in glycerol and examined under a fluorescence microscope equipped with an interference contrast apparatus with a 40× objective (BX 51).

Observation on Binding Behavior of Sperm to the Pvm

Pvm cut into approximately 5 mm in diameter was incubated with 0.2 ml sperm suspension at 1×10^7 sperm/ml in HBSS at 39℃. Sperm binding to the pvm was directly observed under stereomicroscopy.

Results

Effects of Anti-ZP Protein Antibodies on in Vitro Hole Formation in the Pvm by Ejaculated Sperm

To investigate which components of the pvm participate in sperm-egg interaction, we tested the effects of anti-ZP protein antibodies on hole formation in the pvm by an *in vitro* sperm-pvm interaction assay. As shown in Fig. 1, the anti-ZP1 and ZP3 antibodies significantly blocked hole formation by sperm, whereas anti-ZP2, ZP4, ZPD, and normal rabbit serum had no such effect. These results indicate that ZP1 and ZP3 may be involved in sperm-egg interaction in the Japanese quail.

The Effects of ZP1 and ZP3 on Sperm Binding to the Pvm

To clarify whether ZP1 and ZP3 are involved in sperm-pvm binding, we performed a sperm-pvm binding assay in the presence of PTX. In accordance with our previous findings, PTX above 1 μg/ml inhibited hole formation on the pvm by sperm (Fig. 2A). In addition, ZP1-induced AR was inhibited in the presence of PTX (Fig. 2B). The ZP1 and ZP3 proteins were purified from the isolated pvm and the
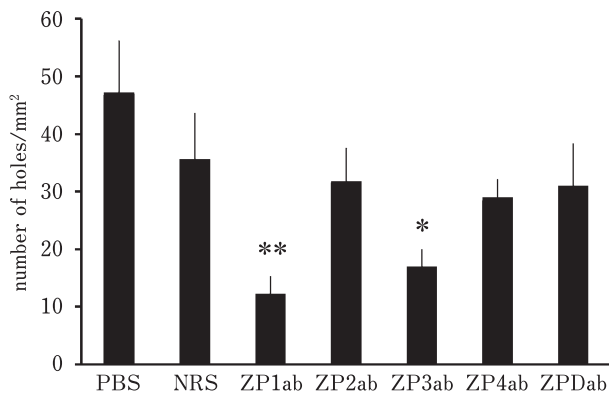

Fig. 1. **Effects of anti-ZP protein antibodies on *in vitro* hole formation in the pvm by ejaculated sperm.** The pvm isolated from the largest follicle was incubated with ejaculated sperm in the presence of the anti-ZP protein antibodies. The holes in the pvm formed by sperm were counted by microscopic observation. Data shown are the mean±S.D. of three experiments. Asterisks indicate a significant difference, *$P<0.05$, **$P<0.01$.

purity of these proteins confirmed by silver staining followed by SDS–PAGE (Fig. 3A). Monomeric ZP1 (97 kDa, lane 4) and ZP3 (35 kDa, lane 2) appeared as single band and we used the purified proteins for the following assay. In the results, the binding of sperm to pvm was blocked in the presence of ZP1 in a dose-dependent manner (Fig. 3A). The addition of purified ZP3 also blocked the sperm-pvm binding, though a higher concentration was required compared to ZP1 (Fig. 3B). These results indicate that ZP1, as well as ZP3, plays a role in the process of sperm binding to the pvm in the Japanese quail.

Interaction of ZP1 and ZP3 Proteins with Sperm

To confirm whether the ZP1 and ZP3 interact with the surface of the sperm, we produced DIG labeled ZP1 and ZP3 and incubated with ejaculated sperm. As shown in Fig. 4, both ZP1 (panel B) and ZP3 (panel A) were immunologically detected on the sperm head. No such intense signal was obtained in the case of the specimen omitting the incubation step with purified ZP proteins (panel C). In addition, light microscopical observation of the pvm incubated with sperm indicated that the sperm appear to bind with pvm through their head (Fig. 5). As shown in the figure, sperm head was tethered to the pvm, whereas their flagellum was free from the pvm.

Discussion

In this study, we performed a sperm-egg interaction assay in the presence of each antibody against five ZP proteins (ZP1, ZP2, ZP3, ZP4 and ZPD). In our results, although anti-ZP1 and ZP3 antibody efficiently blocked hole formation on the pvm (Fig. 1), it is necessary to consider an interpretation of the results. As described in our previous review (Ichikawa *et al*., 2016), there are three possible explanations for this inhibitory effect; 1, the antibody inhibit sperm-egg binding because the antibody covered the ZP1 or ZP3, a binding site for the sperm; 2, the AR of the sperm was blocked by the antibody; 3, the protease responsible for the digestion of pvm is inhibited by the antibody. Therefore, we employed PTX, an inhibitor of AR induction in Japanese quail, in order to discriminate sperm-pvm binding from the other events. As a result, under the conditions employed here, we found that both purified ZP1 and ZP3 significantly inhibited sperm-pvm binding, providing evidence that ZP1 and ZP3 are responsible for sperm-pvm binding. According to the degree of the inhibitory effects of these ZP proteins, ZP1 is considered to possess a higher affinity to the putative sperm surface ZP binding molecules than ZP3. Although the binding partner for ZP1 and ZP3 is not known, Han *et al*. (2010) showed that the O-glycan of ZP3 is important for sperm binding in chicken. We also previously demonstrated that the removal of N-glycan from purified ZP1 by N-glycanase reduced AR-inducing activity (Sasanami *et al*., 2007). To facilitate our understanding of the mechanism for sperm-egg binding in avian species, identification of the binding partner for ZP proteins localized on the sperm surface appears to be indispensable. The question of whether the ZP1 and ZP3 binding to sperm are mediated by identical
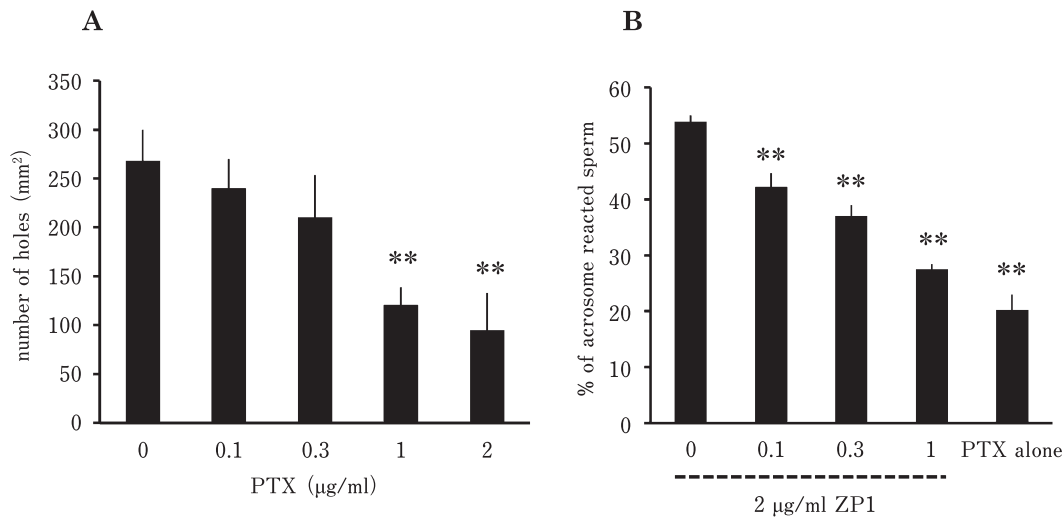
A

B

Fig. 2. **Effects of pertussis toxin on sperm perforation.** (A) The pvm isolated from the largest follicle was incubated with ejaculated sperm in the presence of pertussis toxin (PTX; 0, 0.1, 0.3, 1.0 or 2.0 μg/ml). The holes in the pvm formed by sperm were counted by microscopic observation. Data shown are the mean±S.D. of three experiments. Asterisks indicate a significant difference, ** $P<0.01$. (B) Effects of PTX on the induction of acrosome reaction. The ejaculated sperm were incubated with 2 μg/ml purified ZP1 in the presence or absence of various concentration of PTX (0, 0.1, 0.3 or 1 μg/ml) for 10 min. For control experiment, spermatozoa were incubated with 1 μg/ml PTX alone. The acrosomal status of the sperm was examined under microscope. Data shown are the mean±S.D. of three experiments. Asterisks indicate a significant difference, ** $P<0.01$.

or separate molecules remains to be solved in future studies. We consider that the avian egg is a suitable model for sperm-egg binding, especially using biochemical approaches, because the avian egg is large enough to purify plentiful amounts of ZP proteins.

In the chicken, it is reported that sperm perforation prefers to occur in the germinal disk region (Birkhead *et al*., 1994; Wishart, 1997). Although the cause of this phenomenon is not clear, there is a possibility that the ZP proteins are responsible. ZP2 is expressed in the oocyte of immature follicles and exists in the pvm as a minor component in Japanese quail (Kinoshita *et al*., 2010). ZP2 in the chicken also accumulates in the egg envelope of immature oocytes but it stays behind in the germinal disk region even after the follicle grows to the largest (Nishio *et al*., 2014). These authors emphasized that this restricted localization of ZP2 on the germinal disk may link to the sperm perforation preference around the germinal disk region. Although the binding properties of ZP2 to sperm have not yet been tested in any birds, it is reasonable to suggest that the restricted localization of ZP2 is responsible for sperm binding in the chicken. In our test, however, we failed to find any inhibitory effect to sperm perforation on the pvm in the presence of anti-ZP2 antiserum (Fig. 1). As mentioned above, ZP2 gene is expressed in immature follicles, and ZP2 protein was under the detection limit in the pvm of mature follicles in quail (Kinoshita *et al*., 2010). This indicates the possibility

that the inefficacy of anti-ZP2 antiserum for sperm perforation may be the consequence due to the absence of functional ZP2 protein in the pvm. Same situation may also be true in the case of ZP4 protein (Serizawa *et al*., 2011). Thus, at this juncture, we have no experimental evidence to discuss the potential role of the ZP2 or ZP4 protein in sperm-pvm binding in quail. In our experimental procedure, we did not discriminate the location of the germinal disk of the oocytes when we sampled the pvm. However, it should be noted that the density of the hole on the germinal disk area of quail fertilized egg was equivalent to those of the equatorial or vegetal pole region (Rabbani *et al*., 2006), indicating the possibility that the mechanism underlying sperm-egg binding in quails may differ from that of chickens. In mice, the blockade of polyspermy after fertilization is mediated by the cleavage of ZP2 by ovastacin, a protease released from cortical granules (Burkart *et al*., 2012). However, unlike in mammalian species, such a ZP2 function may not work because polyspermic fertilization, in which plural spermatozoa penetrate into the egg, occurs in birds (Hemmings and Birkhead, 2015; Mizushima *et al.,* 2014). Although the role of ZP2 during fertilization in Japanese quail is not known, we previously demonstrated a possible interaction of the recombinant ZP2 with ZP3 and that this interaction may lead to the formation of an amorphous structure on the cell surface (Kinoshita *et al*., 2010). This finding demonstrates that the ZP2 protein in the immature oocyte might play a role in pvm
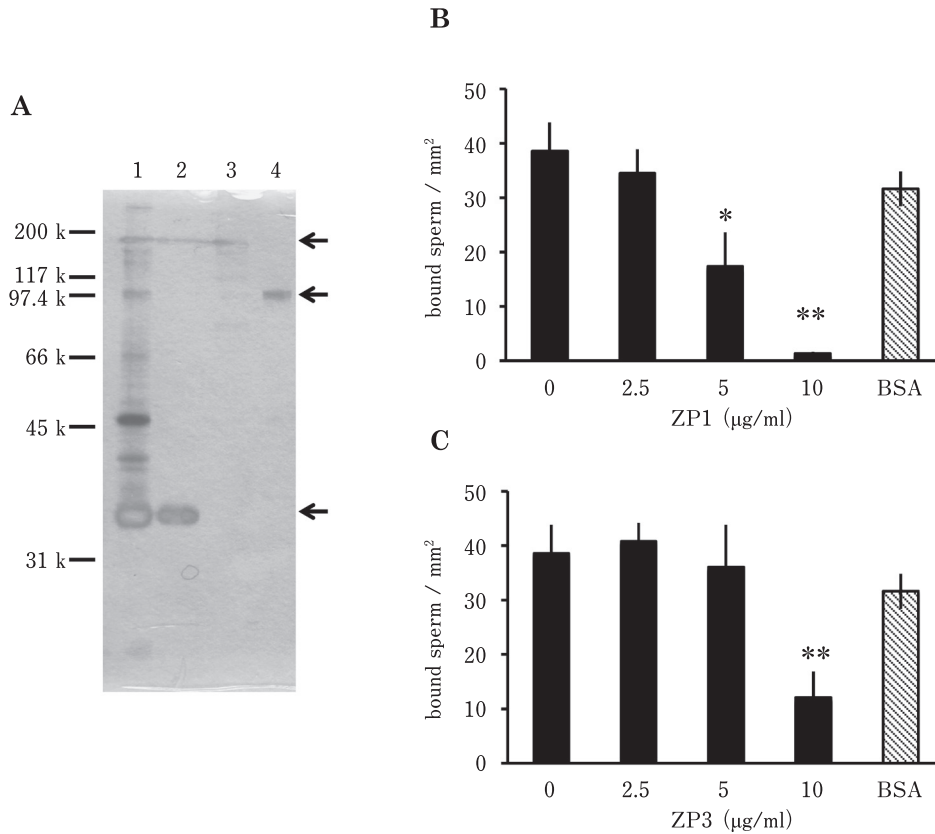
Fig. 3.   **Effects of purified ZP1 and ZP3 on sperm binding to the pvm.** (A) The purified ZP3 (lane 2, 0.1 μg per lane), the purified dimeric ZP1 (lane 3, 0.1 μg per lane), or the purified monomeric ZP1 (lane 4, 0.1 μg per lane) were separated on SDS-PAGE under non-reducing conditions, and detected by silver staining.   The pvm lysate (1 μg per lane) was also separated on SDS-PAGE under non-reducing conditions (lane 1) as a reference.   (B and C) Ejaculated sperm was incubated with pvm in the presence of ZP1 (0, 2.5, 5.0 or 10 μg/m*l*, panel B) or ZP3 (0, 2.5, 5.0 or 10 μg/m*l*, panel C). For negative control, BSA (10 μg/m*l*) was added. The sperm bound on the pvm were counted under microscopic observation.   Data shown are the mean±S.D. of three experiments. Asterisks indicate a significant difference, *$P<0.05$, **$P<0.01$.

formation in the follicles through binding with ZP3. In the case of ZPD, the expression level of the protein increases during follicular development and the immunological studies using anti-ZPD antiserum indicated that ZPD protein exists in the pvm of mature oocytes (Sato *et al*., 2009). Thus the inefficacy of anti-ZPD antiserum on the sperm penetration indicated that ZPD protein does not play a major role in the sperm-egg binding during fertilization in Japanese quail.

Our immunofluorescence study using DIG-labeled ZP1 and ZP3 indicated that ZP1 and ZP3, after incubation with sperm, were detected on the surface of the sperm head. These results coincide with our microscopic observations in which the interaction of sperm with the isolated pvm *in vitro* seems to begin through the sperm head (Fig. 5). Bausek *et al.,* (2004), who tested the binding of purified ZP1 or ZP3 to sperm, also reported that both ZP proteins specifically bind to the tip of the sperm head. It is interesting to note that only

ZP3 binding occurred through interaction with 180 kDa sperm protein in the chicken. We previously demonstrated, by immunological and ultrastructural studies, that 45 kDa acrosin localized on the sperm plasma membrane is responsible for sperm-pvm binding in the Japanese quail, but the binding of acrosin to ZP protein had not been investigated. From this viewpoint, it is very likely that the interaction of plural sperm surface molecules with ZP proteins may participate in sperm-pvm binding during fertilization in birds. Further studies are required to uncover the underlying mechanism of sperm-egg binding in birds.

In conclusion, our results indicate that sperm binding to the pvm is, at least in part, mediated by the interaction of ZP1 and ZP3 with the sperm head at the initial step of fertilization. Additional studies, particularly to identify of binding partner for ZP proteins, are required to improve our understanding of this event.
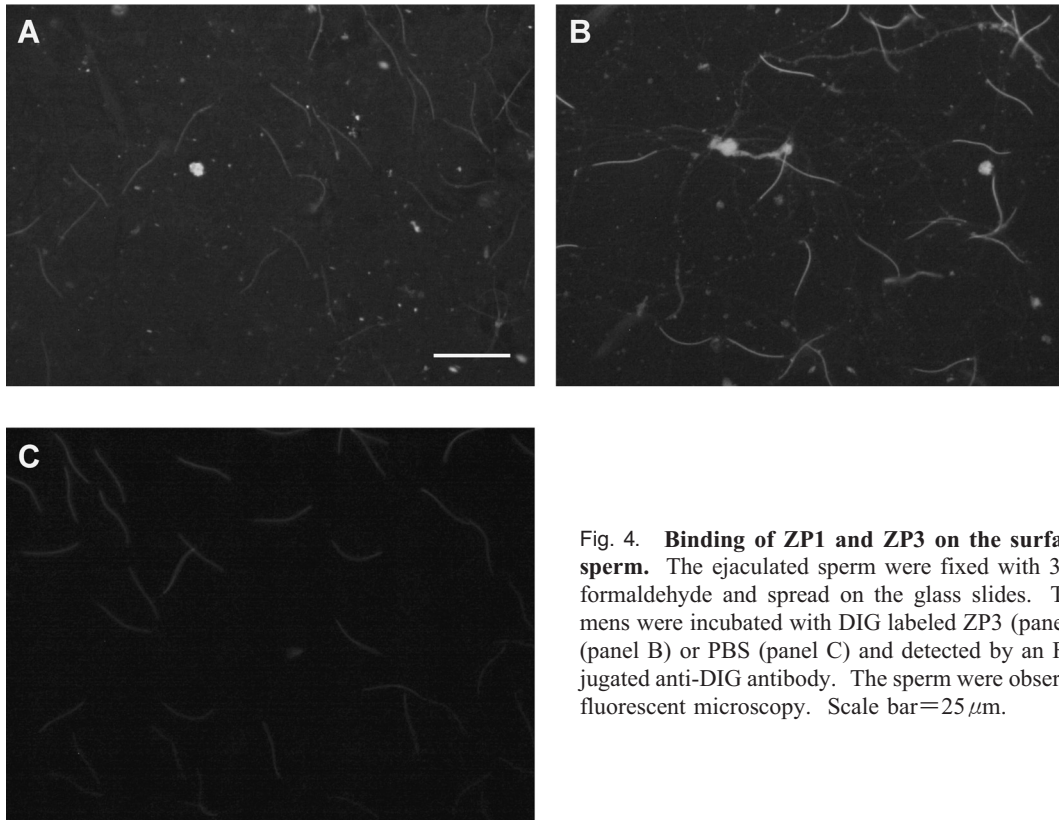
Fig. 4. **Binding of ZP1 and ZP3 on the surface of the sperm.** The ejaculated sperm were fixed with 3.7% (v/v) formaldehyde and spread on the glass slides. The specimens were incubated with DIG labeled ZP3 (panel A), ZP1 (panel B) or PBS (panel C) and detected by an FITC conjugated anti-DIG antibody. The sperm were observed under fluorescent microscopy. Scale bar＝25 μm.
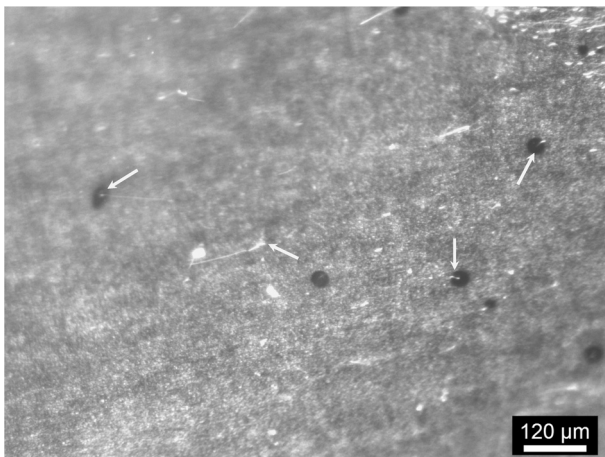

Fig. 5. **Microscopical observation of sperm binding to the pvm.** The pvm isolated from the largest follicle was incubated with ejaculated sperm. Sperm binding to the pvm during incubation was observed under a stereomicroscope. Arrows indicate the sperm bound to the pvm. Scale bar＝120 μm.

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