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Zona Reaction in Porcine Oocytes Fertilized In Vivo and In Vitro as Seen with Scanning Electron Microscopy[1]

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ABSTRACT

Morphological changes in zona pellucidae (ZP) isolated from in vitro-matured (IVM) and ovulated porcine oocytes were compared before or after fertilization in vitro and in vivo, respectively, by using scanning electron microscopy (SEM). The ZP of some ovulated or IVM oocytes and in vivo- or in vitro-fertilized (IVF) zygotes were equally split into two halves while immersed in an enzyme-inhibitor solution, using a surgical blade. After washing, intact and ZP halves were fixed in 1% glutaraldehyde solution in 0.1 M cacodylate buffer, processed, and examined using SEM. The outer surface of ZP in ovulated oocytes had a mesh-like structure. The outer morphology in IVM oocytes was more smooth although the mesh-like structure was still visible at high magnification. In in vivo zygotes and IVM-IVF zygotes, this lysed, mesh-like structure was more obvious. The inner surface of ZP had some small depressions (orifices). The mean number of orifices per 100 μm² of ZP surface was larger in IVM oocytes as compared to ovulated ones. The number of orifices per 100 μm² decreased in IVM-IVF zygotes as compared to IVM oocytes; whereas, in vivo zygotes did not differ from ovulated oocytes. The mean diameter of intact ZP as well as their mean thickness was greater in ovulated oocytes than IVM oocytes. The mean thickness of the ZP was larger in ovulated oocytes than IVM ones. The ZP thickness was larger in zygotes than in in vivo oocytes, whereas that of IVM-IVF zygotes did not differ from that of IVM oocytes. These results indicate that the morphology of ZP and the ZP reaction at sperm penetration appears to be much different between IVM oocytes and ovulated ones.

fertilization, IVF/ART, ovum

INTRODUCTION

Polyspermic penetration is a persistent problem during in vitro production of porcine embryos [1]. In a recently described in vitro fertilization (IVF) system, however, in vitro-matured (IVM) oocytes possessed equal ability to release cortical granules on sperm penetration in vitro, compared to in vivo-matured porcine oocytes [2]. Induction of cortical granule exocytosis of IVM oocytes by calcium ionophore A23187 resulted in prevention of sperm penetration of the zona pellucida (ZP), but not at the oolemmal level [3]. Therefore, an abnormally high incidence of polyspermic penetration in vitro of pig oocytes appears to not be due to delayed or incomplete cortical granule exocytosis but more likely to a delayed ZP reaction and/or simultaneous sperm penetration [4]. Between ovulated and IVM oocytes, the resistance of the ZP to dissolution by pronase was different [2], and the resistance of IVM oocytes increased by exposure to oviductal fluid [5]. However, it has not been clarified whether there is a morphological difference between IVM and ovulated porcine oocytes and whether the ZP reaction is reflected by the origin of the oocytes, although morphological change of the ZP of IVM-IVF bovine zygotes has been analyzed recently [6]. In the present study, morphological changes in the ZP of IVM and ovulated porcine oocytes were observed before or after sperm penetration in vitro and in vivo, respectively, using scanning electron microscopy (SEM). Here we show morphological differences between IVM and ovulated porcine oocytes before and after sperm penetration.

MATERIALS AND METHODS

Media

The medium used for the collection of oocyte-cumulus complexes and washing was modified TL-Hepes-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM Hepes, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 μg/ml gentamicin, and 65 μg/ml potassium penicillin G. The basic maturation medium (OMM37) used was BSA-free North Carolina State University 37 medium [7] supplemented with 0.6 mM cysteine, 5 μg/ml insulin, and 10% (v/v) porcine follicular fluid [8]. The basic medium (mBO) used for IVF was modified BO solution [9], composed of 112.0 mM NaCl, 4.02 mM KCl, 37.0 mM $NaHCO_3$, 0.83 mM $NaH_2PO_4 \cdot 2H_2O$, 1.25 mM Na-pyruvate, 0.50 mM $MgCl_2 \cdot 6H_2O$, 7.50 mM $CaCl_2 \cdot 2H_2O$, 50 μg/ml gentamicin, 6 mg/ml BSA (A6003, Sigma Chemical Co., St. Louis, MO), and 5.0 mM caffeine sodium benzoate. All media were equilibrated at 38.5°C in an atmosphere of 5% CO_2 in air overnight prior to incubation of oocytes. Porcine follicular fluid was prepared from antral follicles (3 to 6 mm in diameter) as described previously [10].

Collection of Ovulated Oocytes and Zygotes

Ovulated oocytes were collected by flushing with modified TL-Hepes-PVA from oviducts of sows (n = 11) slaughtered 44–48 h after the first estrous detection following weaning. Zygotes were collected similarly from mated or artificially inseminated sows slaughtered at the same time. To prevent the zona reaction, ovulated oocytes and

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zygotes were washed three times with an enzyme-inhibitor solution (EIS; modified TL-Hepes-PVA supplemented with 1 mg/ml EDTA, 10 μg/ml lima bean trypsin inhibitor, and 0.1 mM PMSF) and then used for the experiment.

Preparation of Oocytes Matured and Fertilized In Vitro

Ovaries were collected from prepubertal gilts at a local abattoir. Transportation of ovaries to the laboratory was carried out at 23–27°C. Cumulus-oocyte complexes were aspirated through an 18-gauge needle into a disposable 10-ml syringe from antral follicles (3 to 6 mm in diameter) on the surface of ovaries, washed three times with modified TL-Hepes-PVA medium, and then collected in 3 ml of fresh modified TL-Hepes-PVA medium. Fifty cumulus-oocyte complexes with uniform ooplasm and a compact cumulus cell mass were washed three times with OMM37 supplemented with 1 mM dibutyryl cAMP, 0.5 μg/ml FSH, and 0.5 μg/ml LH, and subsequently cultured in 500 μl of the same medium covered with paraffin oil for 20 h at 38.5°C in an atmosphere of 5% CO_2 in air. The complexes were then transferred to 500 μl of OMM37 (without dibutyryl cAMP, FSH, and LH) after washing three times with the same medium. The complexes were cultured for an additional 24 h [10, 11]. After culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and washed carefully three times with mBO. Only oocytes with the first polar body in the perivitelline space were selected under the microscope and considered as IVM oocytes for SEM.

Suspensions of frozen-thawed boar spermatozoa were washed three times with Dulbecco PBS supplemented with 0.1% BSA by centrifugation at 1500 × *g* for 4 min each time. The pellets containing spermatozoa were resuspended at 2×10^6 cells/ml in mBO medium and used for IVF. Thirty denuded oocytes were cocultured with 2×10^6 cells/ml spermatozoa in a 100-μl droplet of mBO under paraffin oil for 10 h at 38.5°C in an atmosphere of 5% CO_2 in air. At 10 h after the start of coculture, IVM-IVF zygotes were centrifuged in an EIS at 15 000 × *g* for 3 min. Only zygotes containing two polar bodies and at least two pronuclei were selected under the microscope and used for the experiment as IVM-IVF zygotes.

Sample Preparation for and Observation by SEM

The ZP of some oocytes or zygotes in each group were split into two halves in the EIS using a surgical blade. After washing, intact and ZP halves were fixed in 1% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) and stored at 4°C for several days until processing. For processing, samples were washed in 67 mM cacodylate buffer for 5 min, postfixed in 2% OsO_4 in the same buffer for 5 min, and then dehydrated in 50% ethanol (for 3 min), 100% ethanol (for 5 min), and 100% acetone (for 3 min). These specimens were critical point-dried with CO_2, mounted on the specimen holder, sputter-coated with platinum/palladium, and examined in a scanning electron microscope, JEOL6320F-SEM. The SEM images were transferred to a PC computer for image analysis (SemAfore, JEOL, Tokyo, Japan) of ZP characters such as the mean number of orifices per 100 μm^2 of ZP surface and the mean diameter of intact ZP specimens.

Statistical Analysis

Statistical analyses of data from at least three sows or four replicate trials were carried out by ANOVA and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program to determine differences between treatments. Data were expressed as mean ± SEM. Probability of $P < 0.05$ was considered to be statistically significant.

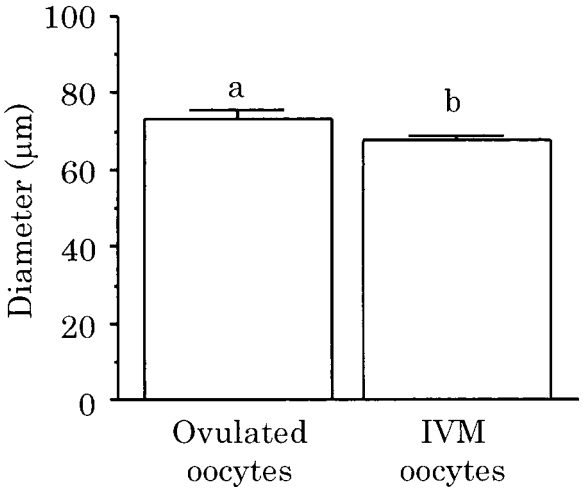

FIG. 1. Mean diameter of the porcine ZP-enclosed oocytes after fixation and preparation for SEM. Different letters above the bars denote statistically significant differences ($P < 0.05$).

RESULTS

In images retrieved via SEM, the mean diameter of intact ZP samples was larger ($P < 0.01$) in ovulated oocytes (72.0 μm, n = 10 from three sows) than IVM ones (67.8 μm, n = 12 from four replications, Fig. 1). The outer surface of ZP in ovulated porcine oocytes had a mesh-like structure with numerous fenestrations (Fig. 2A). The surface was uneven, with a microtrabecular appearance (Fig. 3A). In the in vivo zygotes, however, the spongy-like structure appeared as lysed and the superficial pores were obliterated (Fig. 2B). At higher magnification, the characteristic microtrabecular structure was less conspicuous, although the surface was still somewhat uneven (Fig. 3B). The outer morphology of the ZP of IVM oocytes (Fig. 2C) was characterized as more smooth than that in ovulated oocytes, although the mesh-like structure with fenestrations still existed when observed a higher magnification (Fig. 3C). Furthermore, the surface of the ZP of IVM-IVF zygotes (Fig. 2D) appeared more lysed than that in IVM oocytes (Fig. 2C) and in vivo zygotes (Fig. 2B), and the mesh-like structure had completely disappeared (Fig. 3D). However, the method to select IVM-IVF zygotes by using centrifugation could not separate clearly polyspermic zygotes from IVM-IVF zygotes.

Even following bisection of some ZP using a surgical blade in an EIS, the ZP-morphology (Fig. 4) was similar to that in intact ZP (Fig. 2). As well as with the morphology of the outer side of the ZP, the inner face of the bisected ZP differed among ovulated oocytes, in vivo zygotes, IVM oocytes, and IVM-IVF zygotes (Fig. 5). The inner surface of the ZP was much smoother in ovulated oocytes (Fig. 5A) and in vivo zygotes (Fig. 5B) compared with IVM oocytes (Fig. 5C) and IVM-IVF zygotes (Fig. 5D). At higher magnification, a homogeneous fine network of filaments was observed on the inner surface of the ZP of ovulated oocytes (Fig. 5A), whereas a much more compacted structure was present in IVM oocytes (Fig. 5C). The structure of the inner face of the ZP also changed before and after

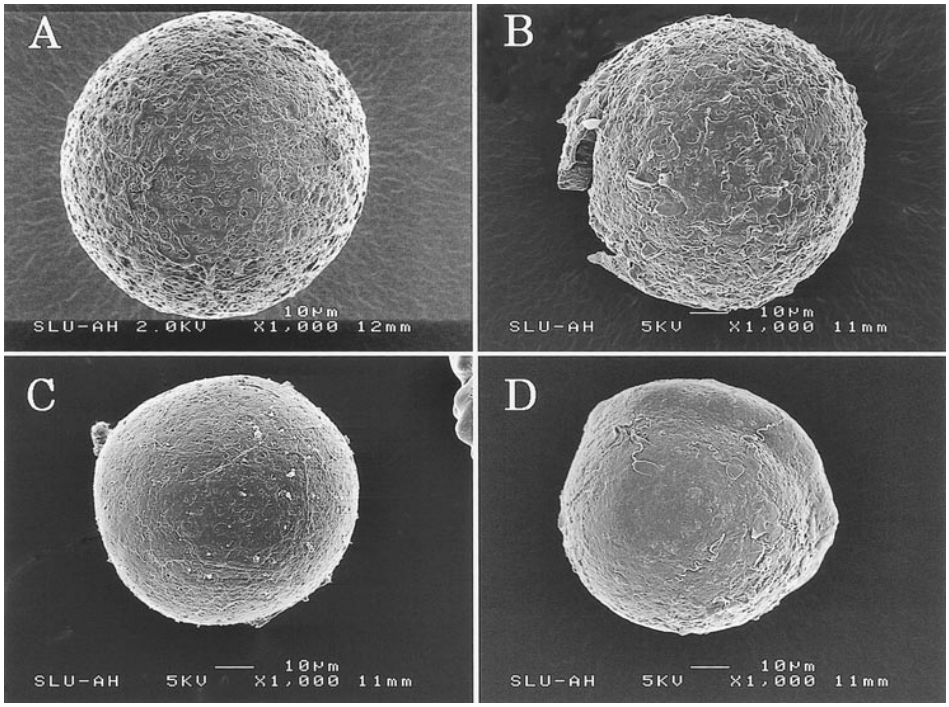

FIG. 2. Scanning electron microscopic images of the ZP of porcine oocytes and zygotes. **A**) Ovulated oocyte. **B**) Zygote collected from oviduct. **C**) An IVM oocyte. **D**) An IVM-IVF zygote.

sperm penetration. In penetrated zygotes, the structure was lysed-like (Fig. 5, B and C) as compared with nonpenetrated oocytes (Fig. 5, A and D). The inner surface of the ZP had some small depressions (orifices). The mean number of orifices per 100 μm^2 of ZP surface was larger ($P < 0.01$) in IVM oocytes (50.6 orifices, n = 19 from four replications) than in ovulated ones (7.1 orifices, n = 21 from five sows, Fig. 6). The number of orifices per 100 μm^2 were lower ($P < 0.01$) in IVM-IVF zygotes (29.6 orifices, n = 18 from four replications) than IVM oocytes; whereas in vivo zygotes (9.6 orifices, n = 19 from three sows) did not differ ($P = 0.71$) from ovulated oocytes (Fig. 6). Foot-process-like structures were found in the orifices of some IVM oocytes but not in ovulated oocytes. The mean thickness of the ZP was larger ($P < 0.01$) in ovulated oocytes (6.68 μm, n = 54 from five sows) than in IVM ones (5.15

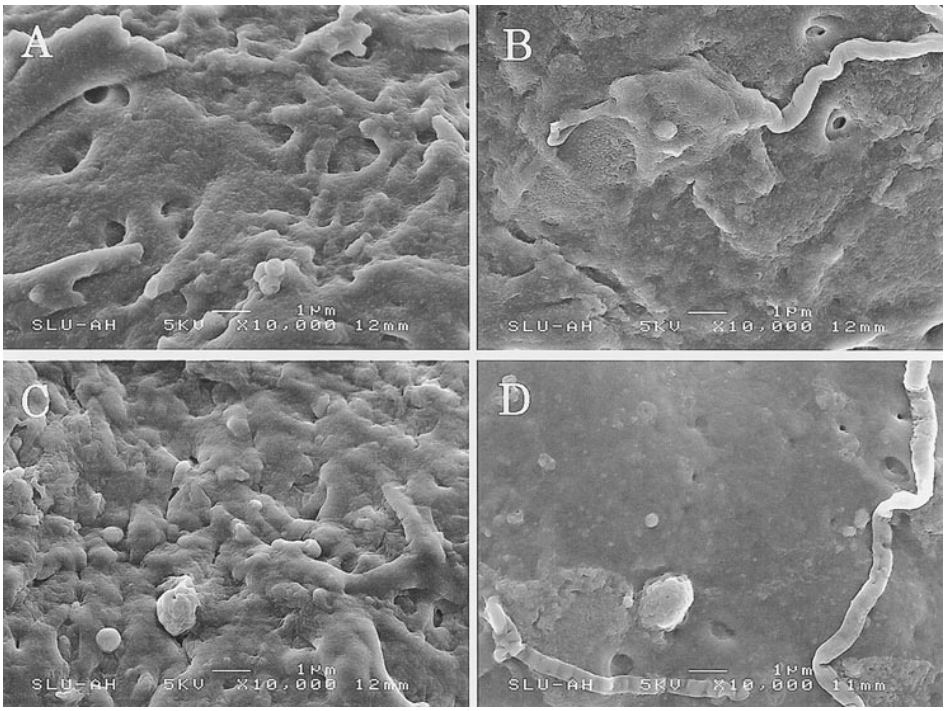

FIG. 3. Scanning electron microscopic images of the outer surface of porcine ZP. **A**) Ovulated oocyte. **B**) Zygote collected from oviduct. **C**) An IVM oocyte. **D**) An IVM-IVF zygote.

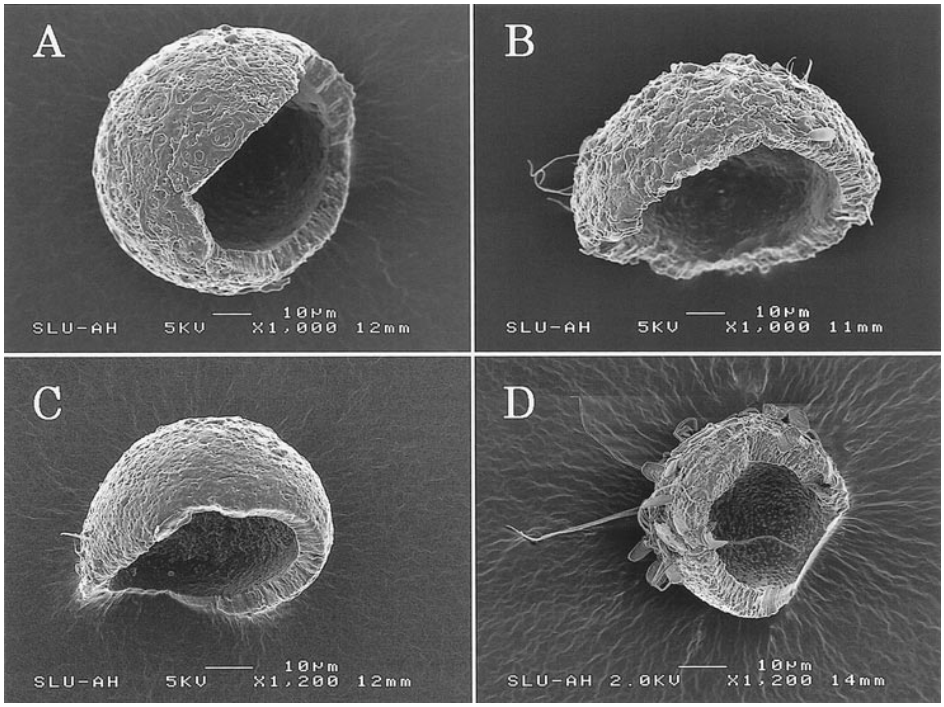

FIG. 4. Scanning electron microscopic images of porcine ZP surgically split into two halves. **A**) Ovulated oocyte. **B**) Zygote collected from oviduct. **C**) An IVM oocyte. **D**) An IVM-IVF zygote.

μm, n = 29 from four replications, Fig. 7). The thickness of the ZP of in vivo oocytes increased ($P < 0.01$) in zygotes (7.48 μm, n = 45 from three sows), whereas that of IVM-IVF zygotes (5.33 μm, n = 43 from four replications) did not differ from that of IVM oocytes (Fig. 7).

DISCUSSION

In the present study, we observed that the outer surface of the ZP of ovulated porcine oocytes had a mesh-like structure with numerous fenestrations and had an uneven surface with a microtrabecular appearance. A similar structure of the outer surface of the ZP has been observed in other mammalian species when studied with SEM [12–17]. On the other hand, we found that the outer morphology of the ZP of IVM oocytes was more compacted and smooth than that in ovulated oocytes. In the human, a smooth ZP was characteristic of immature oocytes or from atretic follicles [18]. In the present study, we selected only oocytes with the first polar body in the perivitelline space as IVM oocytes, showing that meiotically immature oocytes had not been included in the IVM oocyte group. However, there is a possibility that the maturation of the ZP morphology may not have been fully achieved in the IVM oocytes, because cytoplasmic maturation, as determined by developmental competence, is not complete in all oocytes considered ''meiotically mature'' in vitro [19]. In the present study, the mean diameter and thickness of the ZP after treatment for SEM were larger in ovulated oocytes than IVM ones, although these oocytes shrank up to about 50% of their original diameter due to a profound artifact, dehydration during processing for electron microscopy. The ZP of ovulated porcine oocytes contain additional macromolecules [20], the estrogen-dependent oviductal glycoproteins secreted into the oviductal lumen, being of particular interest [21, 22]. These substances in the oviductal intraluminal fluid may be required for the final maturation of the ZP in porcine IVM oocytes. Furthermore, because porcine cumulus-oocyte complexes used for IVM/IVF have been collected by the size of antral follicles, regardless of atresia or not (see Day and Funahashi [19]), there is the possibility that samples for IVM/IVF may contain oocytes from atretic follicles. Therefore, our present results suggest that the compactness of the SEM morphology of the outer ZP of IVM oocytes may be due to a failure in the final maturation of the ZP during in vitro maturation and/or due to the inclusion of follicles undergoing atresia among abattoir-derived ovaries.

The ZP was always split into two halves in the presence of an EIS containing EDTA, lima bean trypsin inhibitor, and PMSF, in order to prevent the occurrence of the ZP reaction. These inhibitors have been used in biochemical analyses of mouse ZP to prevent the ZP reaction during the manipulations of the oocytes [23, 24]. Therefore, bisection in the presence of these inhibitors appears to be a useful method to observe the inner surface of the ZP using SEM. The inner surface of the ZP in ovulated oocytes was smooth with a homogeneous fine network of filaments. Although a similar fine network of filaments has been observed in the ZP of matured human oocytes [16], each filament appeared to be much thicker in the porcine oocytes we examined. As well as with the outer surface, the appearance of the inner face in bisected porcine ZP differed between ovulated and IVM oocytes. The surface of the inner ZP was much smoother in ovulated than IVM oocytes, whereas the fine network of filaments appeared compacted in IVM oocytes. Again, the inner surface of the ZP of IVM oocytes may not have reached final maturation yet and/or may be affected by atresia at collection. The inner surface of the ZP had some small depressions. These orifices corresponded to the spaces where corona cell pseudopodia passed through the ZP to butt against the oolemma, and foot-process-like structures were found in these orifices in some IVM oocytes. We found here that the number of orifices was increased more in IVM than ovulated oocytes, even following fertil-

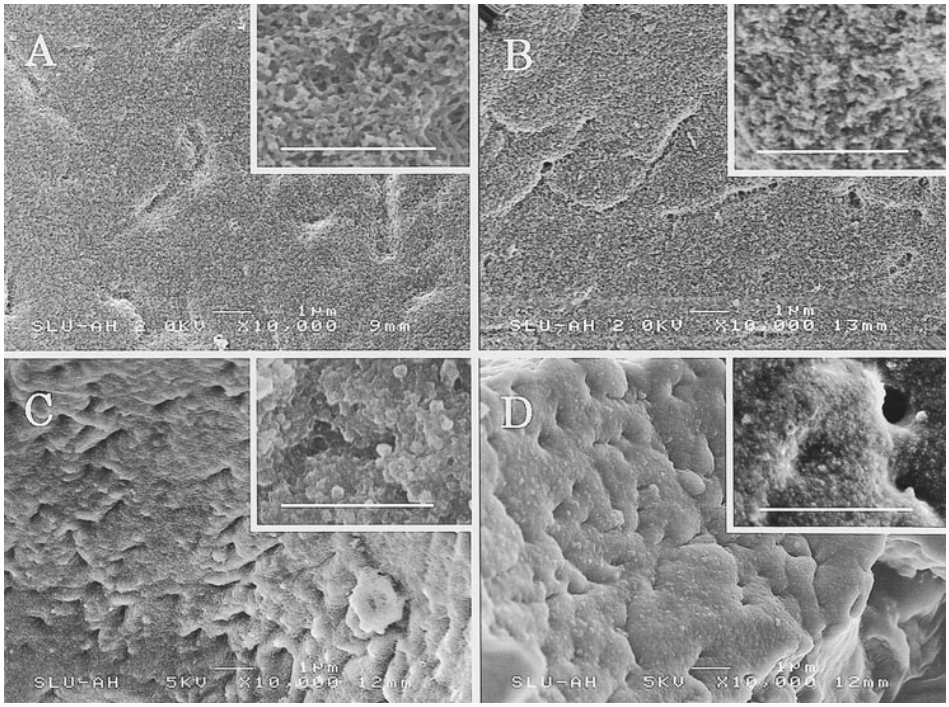

FIG. 5. Scanning electron microscopic images of the inner surface of porcine ZP surgically split into two halves. Greater details are shown at the upper right inset in each image. **A**) Ovulated oocyte. **B**) Zygote collected from oviduct. **C**) An IVM oocyte. **D**) An IVM-IVF zygote.

ization in vitro and in vivo, respectively. Furthermore, the mean number of orifices per IVM oocyte decreased following IVF, but the number did not change in ovulated oocytes during in vivo fertilization. These results suggest that degeneration of the foot processes was delayed and incomplete in IVM oocytes compared with ovulated ones, although transportation of materials via the intercellular coupling of oocyte and corona cells appears to be more active during in vivo maturation (see the review by Moor et al. [25]).

In the present study, we observed that the spongy-like structure of the outer ZP of in vivo zygotes appeared to be lysed, in comparison with the ZP of ovulated oocytes, and that the microtrabecular structure thus present had disappeared. In human fertilized oocytes, superficial pores in the ZP appear to be obliterated by an amorphous material emerging from the inner ZP areas [17]. However, it is still unclear whether the ZP reaction in porcine oocytes occurs solely in either the inner or the outer ZP, because the lysed structure was found both in the deeper parts of the pores and in the superficial microtrabecules. In the present study, furthermore, the morphology of the ZP in IVM oocytes had also changed during IVF, although the outer ZP surface was

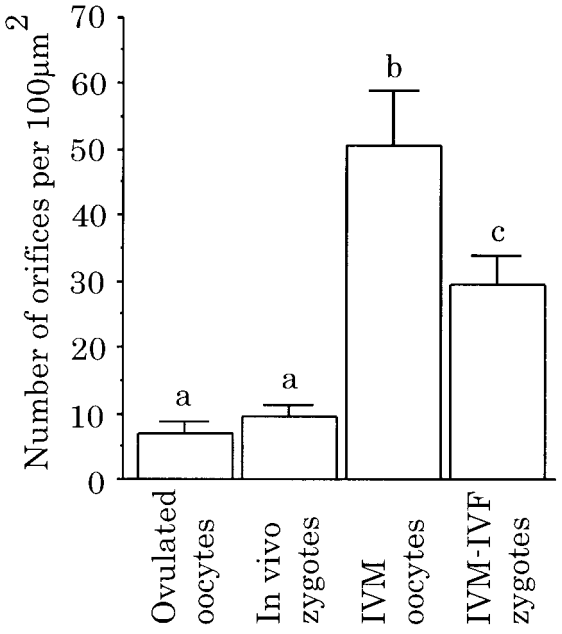

FIG. 6. Mean number of orifices on the inner surface of porcine ZP. Different letters above the bars denote statistically significant differences ($P < 0.05$).

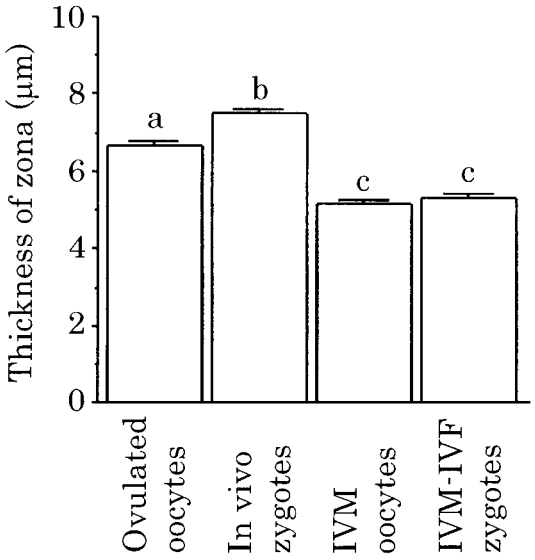

FIG. 7. Mean thickness of porcine ZP after fixation and preparation for SEM. Different letters above the bars denote statistically significant differences ($P < 0.05$).

more lysed in IVM-IVF zygotes compared to zygotes collected from oviducts and the mesh-like structure thus present was not visible any longer. The inner structure of the ZP with a homogeneous fine filament network was also lysed during fertilization, although some differences were observed between IVM/IVF zygotes and in vivo zygotes. Morphological differences in the inner and outer ZP faces between IVM/IVF zygotes and in vivo zygotes may be due to differences in the morphology between IVM oocytes and ovulated ones. The properties and composition of the ZP have been shown to change during IVF of IVM oocytes in a biochemical study [26]. In IVM/IVF zygotes, polyspermic penetration has been a persistent and major problem [1]. The incidence of polyspermy appears to be lower in ovulated oocytes than in IVM ones, if those oocytes were inseminated in a medium containing a high concentration of calcium and the absence of fetal bovine serum [2]. In a physiological situation, the defense mechanism against polyspermy in pig oocytes is achieved mainly by the ZP reaction following the exocytosis of cortical granules that prevents against sperm penetration [27]. According to a recent study by Wang et al. [2], however, the mean numbers of cortical granules beneath the plasma membrane before and after sperm penetration were not different between IVM and ovulated oocytes. In the present study, cortical granules were regularly distributed as beads on a string in the IVM oocytes (data not shown). Polyspermic penetration in vitro is not due to a delayed or incomplete cortical granule exocytosis but more likely to a delayed or incomplete ZP reaction and/or to simultaneous sperm penetration [4]. In the present study, we demonstrated that a morphological ZP reaction was achieved even in IVM porcine oocytes following IVF. A mesh-like structure with numerous fenestrations of the ZP in ovulated oocytes may play a role in reducing the incidence of simultaneous sperm penetration. Furthermore, we observed that the thickness of in vivo oocytes increased in zygotes but did not change during IVF of IVM oocytes. Therefore, the ZP reaction of IVM-IVF zygotes may be far from complete. The macromolecular differences between the oocyte and zygote ZP are caused by the addition of macromolecules to the ZP as the oocyte transits the oviduct [20], suggesting that estrogen-dependent oviductal glycoproteins [21, 22] may contribute to the completeness of the ZP reaction.

In conclusion, our results indicate that the morphology of ZP and the ZP reaction at sperm penetration differ between IVM oocytes and ovulated ones. Oviductal glycoproteins may contribute to complete a final maturation and penetration-dependent reaction of the porcine ZP. Although recent developments in porcine IVM systems have made possible the efficient production of piglets from offal oocytes (see review by Funahashi and Day [1]), the morphology and the reaction of the pig ZP appear not to be the same as that of in vivo oocytes yet.

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