

# Transmission electron microscopy studies of the zona reaction in pig oocytes fertilized *in vivo* and *in vitro*

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The aim of this study was to determine the ultrastructure of cross-sectioned zonae pellucidae of *in vitro*-matured and ovulated pig oocytes before or after sperm penetration *in vitro* and *in vivo*, respectively. The *in vitro* and *in vivo* (ovulated) oocytes and zygotes (fertilized *in vitro* and *in vivo*) were fixed with glutaraldehyde either directly or after pretreatment with ruthenium red and saponin, processed and then examined using transmission electron microscopy. The thickness of the zona pellucida, as measured on the section of the specimens with largest diameter fixed with glutaraldehyde, differed between the *in vivo* ( $9.19 \pm 0.47 \mu\text{m}$ ) and *in vitro* ( $5.95 \pm 0.51 \mu\text{m}$ ) oocytes. The *in vivo* oocytes had a rather thick external mesh-like structure, whereas it was much thinner in the *in vitro* oocytes. This mesh-like external rim was less apparent in both *in vivo* and *in vitro* zygotes. Obvious differences in the density of the lattice formed by the fixed zonae pellucidae were visible between the outer and inner

(ad-olemmal) zonae. The outer area always formed a concentrically arrayed fibrillar network, whereas the inner area showed a much more compact, trabecule-like mesh. However, both areas, but particularly the outer network, were much more compacted after the zona reaction. Clear differences in the degree of fibrillar aggregation of the inner zona area were also observed between *in vitro* and *in vivo* zygotes, being much higher in the latter. This fibrillar network was more clearly visible in the zygotes pretreated with ruthenium red and saponin; the *in vitro* zygotes had a fibrillar, radially oriented set of parallel fibrils, whereas it was much more aggregated and trabecule-like in the *in vivo* zygotes. These results demonstrate that the fine structure of the zona pellucida and the zona reaction at sperm penetration differ between pig oocytes fertilized *in vivo* and *in vitro*. Moreover, the ultrastructure of the outer and inner pig zonae pellucidae has a different network organization.

## Introduction

The zona pellucida is a glycoprotein coat that protects oocytes and embryos from physical damage and also regulates sperm penetration. At fertilization, the entry of multiple spermatozoa into the oocyte is prevented by the zona pellucida. However, polyspermic penetration has been a major obstacle of *in vitro* fertilization (IVF) of *in vitro*-matured oocytes in pigs. Wang *et al.* (1998a,b, 1999) reported that the abnormally high incidence of polyspermic penetration of pig oocytes *in vitro* is not due to delayed or incomplete cortical granule exocytosis but more probably to a delayed zona pellucida reaction or simultaneous sperm penetration or both. Funahashi *et al.* (2000) demonstrated that the surface morphology of the zona pellucida and the zona reaction at sperm penetration differed between *in vitro*-matured and ovulated oocytes, as detected by

scanning electron microscopy (SEM). In that study, the outer morphology of ovulated oocytes was a mesh-like structure, whereas that of *in vitro*-matured oocytes was much smoother. Wang *et al.* (1998a) reported that the resistance of the zona pellucida to dissolution by pronase was different between ovulated and *in vitro*-matured oocytes and Kim *et al.* (1996) reported that the resistance of *in vitro*-matured oocytes was increased by exposure to oviductal fluid. However, it has not been clarified whether the morphological differences between the zonae pellucidae of *in vitro*-matured and ovulated pig oocytes are only at the surface and, moreover, whether the zona reaction reflects the origin of the oocytes.

In the present study, changes in the ultrastructure of cross-sectioned zonae pellucidae of *in vitro*-matured and ovulated (*in vivo*) pig oocytes were observed before or after sperm penetration *in vitro* and *in vivo*, respectively, using transmission electron microscopy (TEM). The differences in the inner structure of zonae pellucidae between *in vitro* and ovulated pig oocytes before and after sperm penetration are described.

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## Materials and Methods

### Reagents

All reagents used were purchased from Sigma Chemical Co (St Louis, MO).

### Media

The medium used for the collection of cumulus–oocyte complexes (COCs) and washing was modified TL–Hepes–PVA medium composed of 114 mmol NaCl l<sup>-1</sup>, 3.2 mmol KCl l<sup>-1</sup>, 2 mmol NaHCO<sub>3</sub> l<sup>-1</sup>, 0.34 mmol KH<sub>2</sub>PO<sub>4</sub> l<sup>-1</sup>, 10 mmol sodium lactate l<sup>-1</sup>, 0.5 mmol MgCl<sub>2</sub>·6H<sub>2</sub>O l<sup>-1</sup>, 2 mmol CaCl<sub>2</sub>·2H<sub>2</sub>O l<sup>-1</sup>, 10 mmol Hepes l<sup>-1</sup>, 0.2 mmol sodium pyruvate l<sup>-1</sup>, 12 mmol sorbitol l<sup>-1</sup>, 0.1% (w/v) polyvinylalcohol, 25 µg gentamicin ml<sup>-1</sup> and 65 µg potassium penicillin G ml<sup>-1</sup>. The basic maturation medium (OMM37) used was BSA-free North Carolina State University 37 medium (Petters and Wells, 1993) supplemented with 0.6 mmol cysteine l<sup>-1</sup>, 5 µg insulin ml<sup>-1</sup> and 10% (v/v) pig follicular fluid (Funahashi *et al.*, 1997). The basic medium used for IVF was modified BO solution (Brackett and Oliphant, 1975), composed of 112.0 mmol NaCl l<sup>-1</sup>, 4.02 mmol KCl l<sup>-1</sup>, 37.0 mmol NaHCO<sub>3</sub> l<sup>-1</sup>, 0.83 mmol NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O l<sup>-1</sup>, 1.25 mmol sodium pyruvate l<sup>-1</sup>, 0.50 mmol MgCl<sub>2</sub>·6H<sub>2</sub>O l<sup>-1</sup>, 7.50 mmol CaCl<sub>2</sub>·2H<sub>2</sub>O l<sup>-1</sup>, 50 µg gentamicin ml<sup>-1</sup>, 6 mg BSA ml<sup>-1</sup> (A6003; Sigma) and 5 mmol caffeine sodium benzoate l<sup>-1</sup>. All media were equilibrated at 38.5°C in an atmosphere of 5% CO<sub>2</sub> in air overnight before incubation of the oocytes. Pig follicular fluid was prepared from antral follicles (3–6 mm in diameter) as described by Funahashi *et al.* (1994).

### Collection of ovulated oocytes and zygotes matured and fertilized in vivo

Immediately after weaning, multiparous crossbred (Yorkshire × Landrace) sows ( $n = 11$ ) were put into individual pens and checked twice a day for signs of oestrus in the presence of a boar. Ovulated oocytes and resulting zygotes were collected surgically with TL–Hepes–PVA at 44–48 h after detection of standing oestrus from control and inseminated sows, respectively. Only eggs with many spermatozoa binding to the zona pellucida (as observed by microscopy) were selected and considered as zygotes. Ovulated oocytes and zygotes were washed three times with an enzyme inhibitor solution (modified TL–Hepes–PVA supplemented with 1 mg EDTA ml<sup>-1</sup>, 10 µg lima bean trypsin inhibitor l<sup>-1</sup> and 0.1 mmol phenylmethylsulphonyl fluoride l<sup>-1</sup>) and used for the experiment.

### Preparation of in vitro-matured oocytes and zygotes matured and fertilized in vitro

Ovaries were collected from prepubertal gilts at a local abattoir. The ovaries were transported to the laboratory at 23–27°C. COCs were aspirated through an 18-gauge needle into a disposable 10 ml syringe from antral follicles

(3–6 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. COCs with uniform ooplasm and a compact cumulus cell mass ( $n = 50$ ) were washed three times with OMM37 supplemented with 1 mmol dibutyl cAMP l<sup>-1</sup>, 0.5 µg FSH ml<sup>-1</sup> and 0.5 µg LH ml<sup>-1</sup>, 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<sub>2</sub> in air. The complexes were transferred to 500 µl OMM37 (without dibutyl cAMP, FSH and LH) after washing three times with the same medium. The complexes were cultured for an additional 24 h (Funahashi and Day, 1993; Funahashi *et al.*, 1994). After culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and washed carefully three times with modified BO medium.

Frozen–thawed boar sperm suspensions were washed three times with Dulbecco's PBS supplemented with 0.1% (w/v) BSA by centrifugation at 1500 g for 4 min each time. The pellets containing spermatozoa were resuspended at  $2 \times 10^6$  cells ml<sup>-1</sup> in modified BO medium and used for IVF. Denuded oocytes ( $n = 30$ ) were cocultured with  $1 \times 10^6$  cells ml<sup>-1</sup> in a 100 µl droplet of modified BO medium under paraffin oil for 10 h at 38.5°C in an atmosphere of 5% CO<sub>2</sub> in air. At 10 h after the start of coculture, *in vitro*-matured and fertilized (IVM–IVF) eggs were centrifuged in an enzyme inhibitor solution at 15 000 g for 3 min. Only eggs containing two polar bodies and at least two pronuclei were selected under a microscope and used for the experiment. Specimens of *in vitro*-matured oocytes and IVM–IVF ova were collected from four cycles of *in vitro* maturation and fertilization.

### Sample preparation and observation by transmission electron microscopy

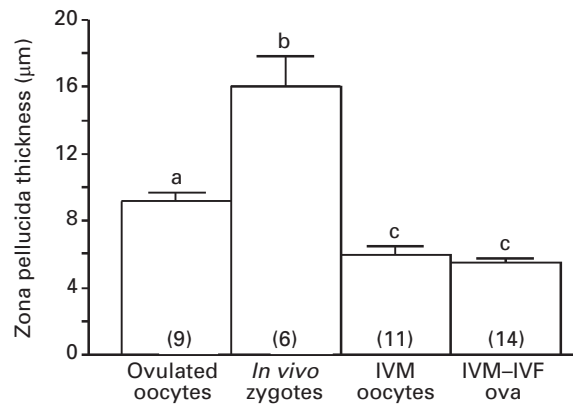
*In vivo*-matured oocytes (ovulated oocytes) and *in vivo* zygotes, as well as the *in vitro*-matured oocytes and IVM–IVF ova with two polar bodies and at least two pronuclei (*in vitro* zygotes) were fixed in 1% (v/v) glutaraldehyde solution in 0.1 mol cacodylate buffer l<sup>-1</sup> (pH 7.2) and stored in the same solution at 4°C until processed. Some of the samples were incubated in a solution of 1% (w/v) ruthenium red and 0.02% (w/v) saponin in 0.1 mol cacodylate buffer l<sup>-1</sup> and fixed in 1% (v/v) glutaraldehyde solution in 0.1 mol cacodylate buffer l<sup>-1</sup> to increase the likelihood of disclosing the fibrillar networks of the zonae pellucidae (Familiari *et al.*, 1992). After postfixation for 5 min in 2% (w/v) osmium tetroxide in the same buffer, continuous sections of oocytes and zygotes were processed conventionally for TEM. Therefore, zona pellucida thickness of the specimen with the largest diameter should represent the correct thickness of the zona pellucida. The thickness should be greater in specimens with smaller diameter. Counter-stained ultrathin sections with a maximum diameter of specimen were examined in a Philips 420 transmission electron microscope at 80 kV.

### Statistical analysis

Statistical analyses of zona pellucida thickness using 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 program (Abacus Concepts Inc., Berkeley, CA) to determine treatment differences. Data are expressed as mean  $\pm$  SEM. The level of significance was  $P < 0.05$ .

### Results

In images retrieved via TEM, the mean thickness of zona pellucida samples was greater ( $P < 0.01$ ) in ovulated oocytes ( $9.19 \pm 0.47 \mu\text{m}$ ;  $n = 9$  samples from five sows) than in *in vitro*-matured oocytes ( $5.95 \pm 0.51 \mu\text{m}$ ;  $n = 11$  samples from four replicates; Fig. 1). The thickness of the zona pellucida was greater ( $P < 0.01$ ) in *in vivo* zygotes ( $16.04 \pm 1.80 \mu\text{m}$ ;  $n = 6$  samples from three sows) than in ovulated oocytes, whereas the thickness of the zona pellucida of *in vitro* zygotes ( $5.51 \pm 0.24 \mu\text{m}$ ;  $n = 14$  samples from four replicates) did not differ from that of *in vitro*-matured oocytes (Fig. 1). The ovulated oocytes had a rather thick external mesh-like structure (about one-third of the zona pellucida; Fig. 2a,b), whereas the structure was much thinner in the *in vitro*-matured oocytes (Fig. 3a,b). This mesh-like external rim was less apparent in both *in vivo* and *in vitro* zygotes. More remnants of foot processes of the corona cells were found inside the zona pellucida, especially in the external part, in *in vitro*-matured oocytes (Fig. 3a) and *in vitro* zygotes (Fig. 4a), compared with ovulated oocytes (Fig. 2a) and *in vivo* zygotes (Fig. 5a). As expected, cortical granules were present under the oolemma in both ovulated (Fig. 2a,c) and *in vitro*-matured oocytes (Fig. 3a,c) but were completely absent in *in vivo* (Fig. 5a,c) and *in vitro* (Fig. 4a,c) zygotes. In addition, zona pellucida sperm tracks were evident in the *in vivo* (Fig. 5b) and *in vitro* (Fig. 4b) zygotes. Obvious differences in the density of the lattice formed by the fixed zonae pellucidae were visible between the outer and inner (ad-oolemmal) zona pellucida. The outer area always formed a concentrically arrayed fibrillar network, mainly parallel to the surface of cytoplasm (Figs 2d and 3d), whereas the inner area showed a much more compact, trabecule-like mesh (Figs 2e and 3e). The fibrillar network in the outer zona pellucida was more coarse in ovulated oocytes (Fig. 2d) than in the *in vitro*-matured oocytes (Fig. 3d). Both areas, but particularly the outer network, were much more compact after the zona pellucida reaction (Figs 4d and 5d). The concentrically arrayed fibrillar network was not observed in *in vivo* zygotes (Fig. 5d), but was visible, albeit incomplete, in *in vitro* zygotes (Fig. 4d). Clear differences in the degree of fibrillar aggregation of the inner zona pellucida area were observed between *in vivo* (Fig. 5e) and *in vitro* (Fig. 4e) zygotes: fibrillar aggregation was much higher in the former. This fibrillar network was more clearly visible in the zygotes pre-treated with ruthenium red and

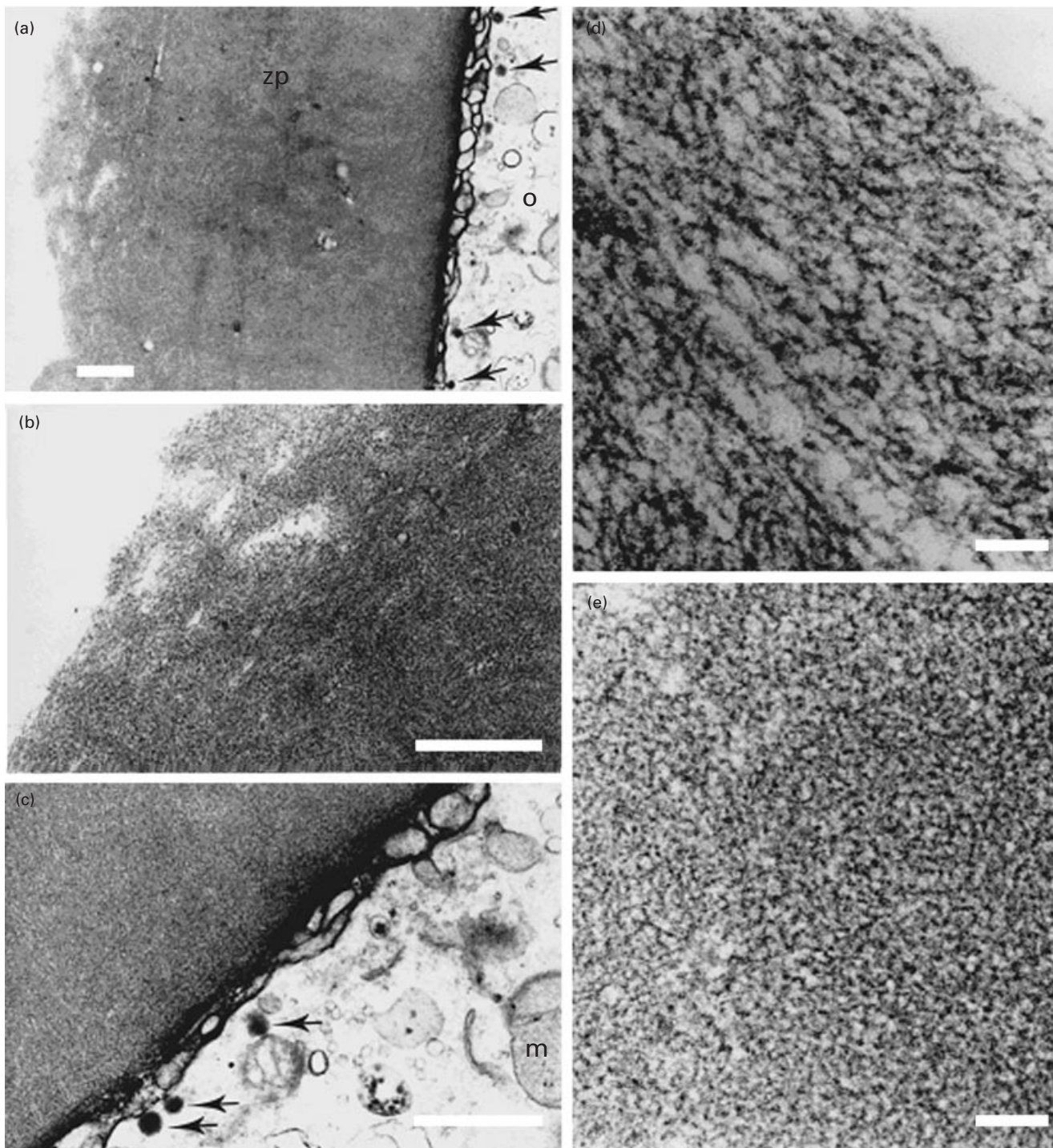


**Fig. 1.** Mean thickness of pig zonae pellucidae after fixation and preparation for transmission electron microscopy. *In vivo* zygotes: zygotes resulting from fertilization of ovulated oocytes; IVM oocytes: oocytes matured *in vitro*; IVM-IVF ova: zygotes resulting from *in vitro* maturation and fertilization. <sup>abc</sup>Values with different letters are significantly different ( $P < 0.05$ ). Numbers in parentheses show the number of zonae pellucidae examined in each group.

saponin for a shorter period (Fig. 6 a–f). The *in vitro* zygotes had a conspicuous fibrillar, radially orientated set of parallel fibrils in the inner area of the zona pellucida (Fig. 6 a,c), whereas the fibrillar network in the *in vivo* zygotes (Fig. 6 b,d) was much more aggregated and trabecule-like (Fig. 6c,d). The outer part of the zonae pellucidae was less arranged in both cases, partially losing these orientations (Fig. 6e,f).

### Discussion

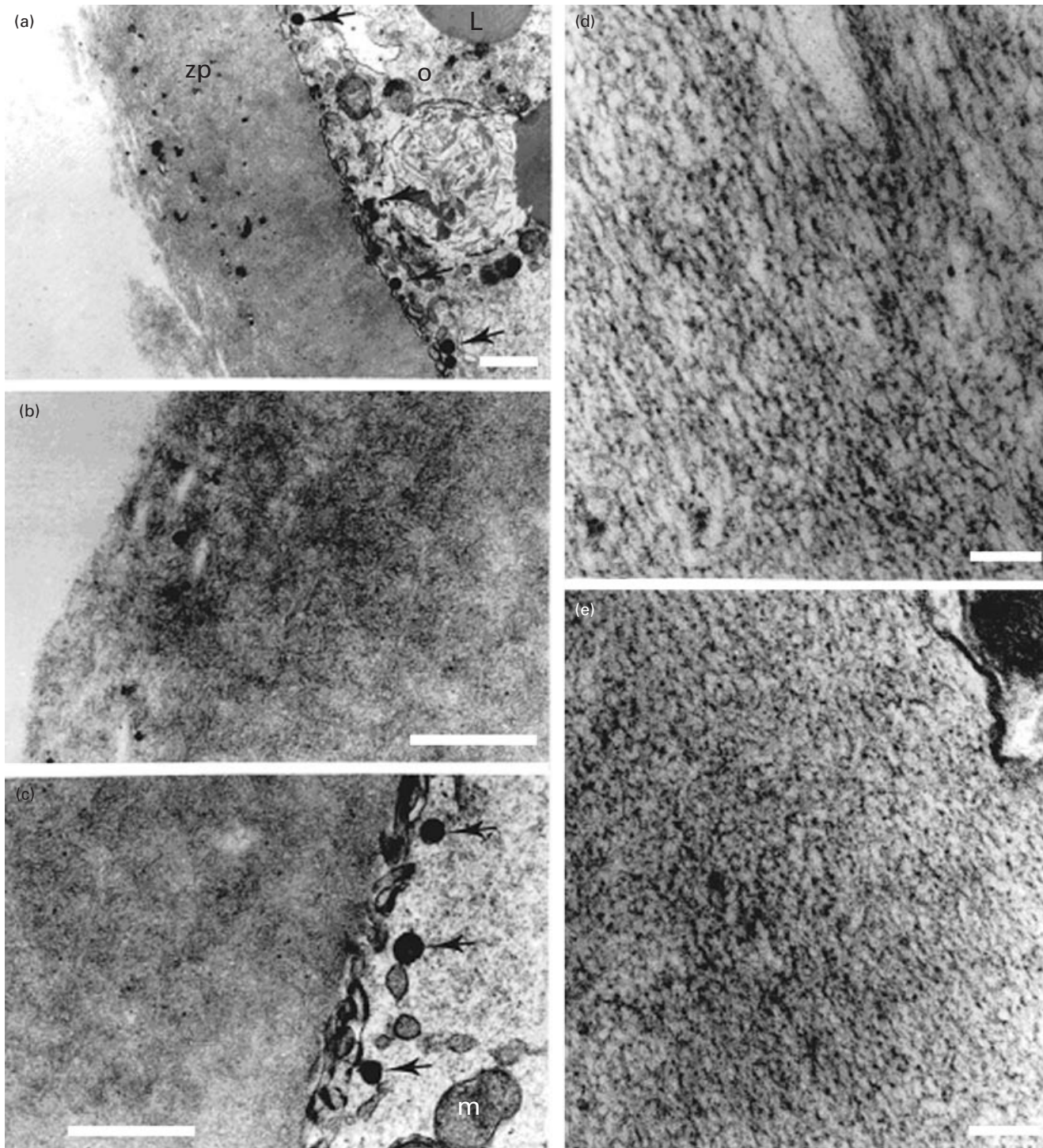
A heterogeneous structure in pig zona pellucida with differences between the external and internal surface has been observed using light microscopy (Dunbar *et al.*, 1980) and TEM (Dietl and Czuppon, 1984). The aim of the present study was to clarify whether the fine cross-sectioned structure of pig zonae pellucidae differs between ovulated and *in vitro*-matured oocytes, and how those structures change before and after the zona reaction. Differences in morphology were observed between the external and internal structure of the zona pellucida in both *in vitro*-matured and ovulated oocytes. Furthermore, a rather thick external mesh-like structure was found in ovulated oocytes compared with *in vitro*-matured oocytes. Using SEM, Funahashi *et al.* (2000) observed that the outer surface of the zona pellucida of ovulated pig oocytes has a mesh-like structure with numerous fenestrations and an uneven surface with a micro-trabecular appearance, whereas the outer morphology of the zona pellucida of *in vitro*-matured oocytes is more compacted and smooth. The difference in external morphology of cross-sectioned zonae pellucidae observed in the present study reflects the surface structure demonstrated previously with SEM. In the present study, traces of foot processes of the corona cells were more common in the external zona pellucida in *in vitro*-matured



**Fig. 2.** Transmission electron microscope images of the zonae pellucidae of ovulated pig oocytes. (a) A part of the zona pellucida (zp). o: ooplasm. Arrows show cortical granules. (b) Outer part of the zona pellucida. (c) Inner part of zona pellucida. m: mitochondrion. Arrows show cortical granules. (d) Outer part of zona pellucida. (e) Inner part of zona pellucida. Scale bars represent 1  $\mu\text{m}$  (a,b,c) or 100 nm (d,e).

oocytes and *in vitro* zygotes compared with ovulated oocytes and zygotes. These results indicate that maturation of the zona pellucida may not have been achieved fully in the *in vitro*-matured oocytes, supporting our previous

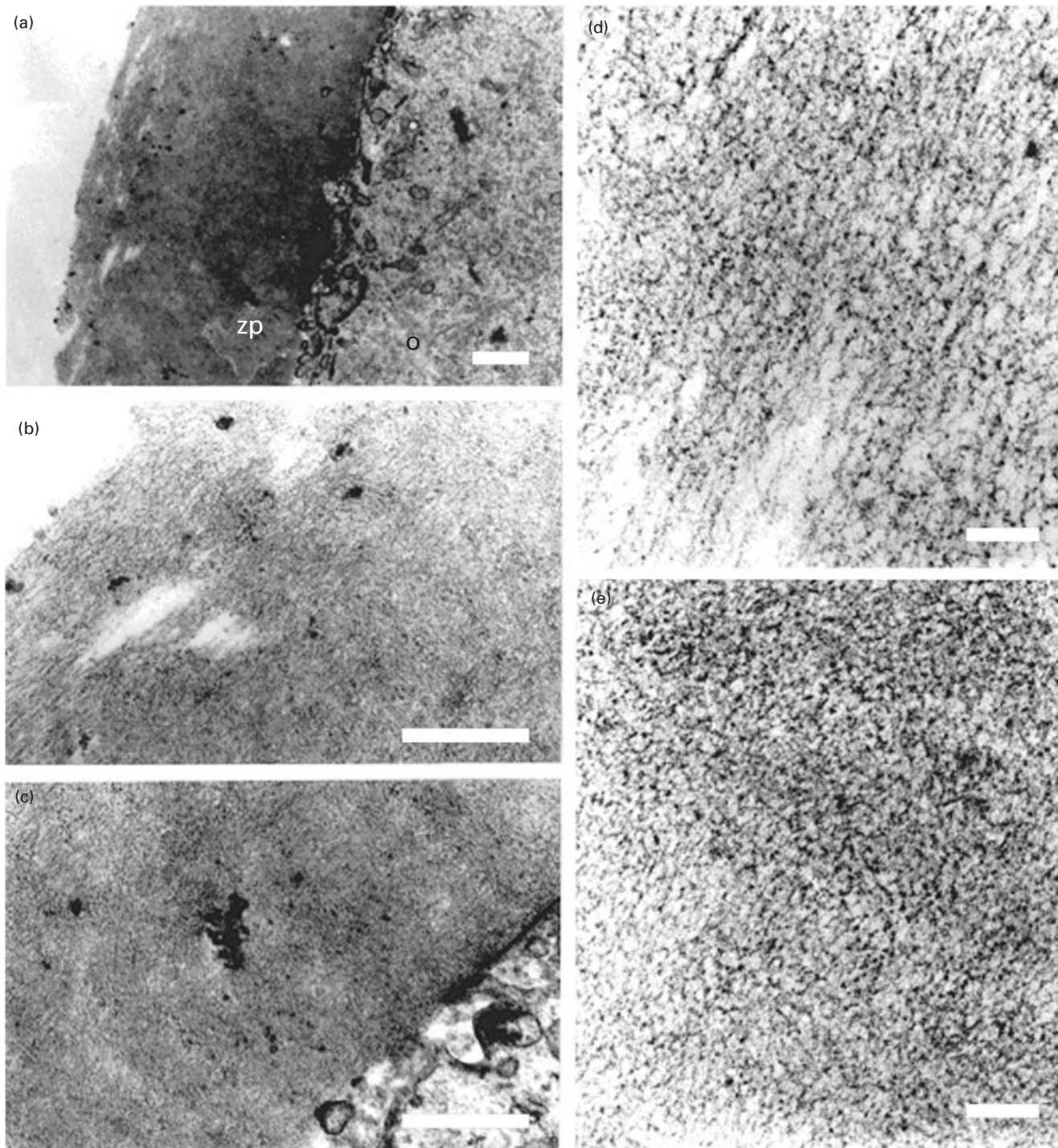
observation by SEM (Funahashi *et al.*, 2000). Although current systems for *in vitro* production of pig embryos can result in development of > 40% of embryos to the blastocyst stage and can produce normal piglets (Funahashi and Day,



**Fig. 3.** Transmission electron microscope images of the zonae pellucidae of *in vitro*-matured pig oocytes. (a) A part of the zona pellucida (zp). o: ooplasm; L: lipid droplet. Arrows show cortical granules. (b) Outer part of zona pellucida. (c) Inner part of zona pellucida. m: mitochondrion. Arrows show cortical granules. (d) Outer part of zona pellucida. (e) Inner part of zona pellucida. Scale bars represent 1  $\mu$ m (a,b,c) or 100 nm (d,e).

1997; Day, 2000), maturation of zona pellucida in pig oocytes still appears to be insufficient *in vitro*. Therefore, *in vitro*-matured oocytes appear to have undergone insufficient morphological maturation of zonae pellucidae.

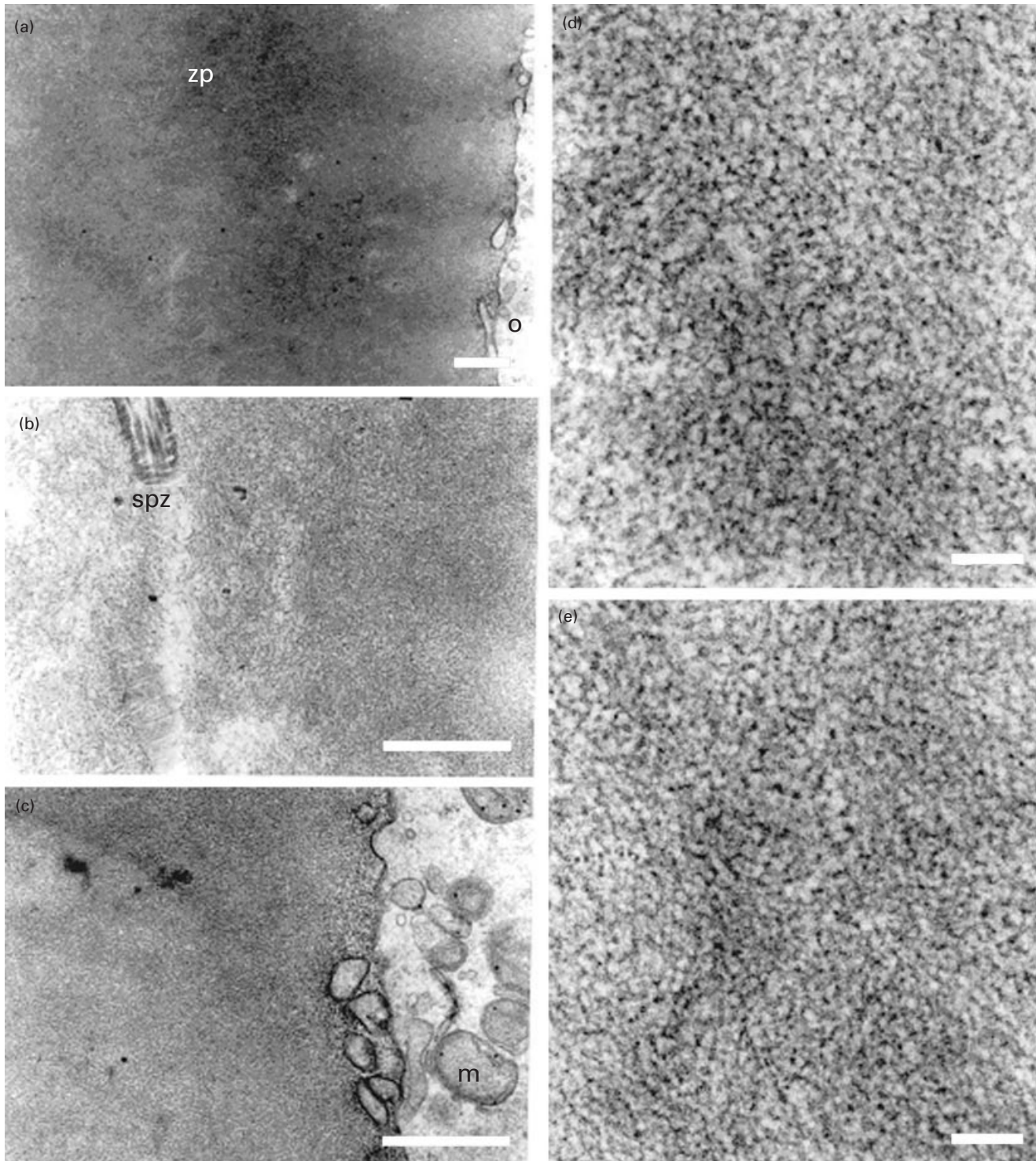
At a high magnification, it was found that a concentrically arrayed fibrillar network, mainly parallel to the surface of cytoplasm, was always observed in the outer area of the zona pellucida in both ovulated and *in vitro*-



**Fig. 4.** Transmission electron microscope images of the zonae pellucidae of an *in vitro*-fertilized pig ovum. (a) A part of the zona pellucida (zp). o: ooplasm. (b) Outer part of zona pellucida. (c) Inner part of zona pellucida. (d) Outer part of zona pellucida. (e) Inner part of zona pellucida. Scale bars represent 1  $\mu\text{m}$  (a,b,c) or 100 nm (d,e).

matured oocytes. However, the fibrillar network was coarser in ovulated oocytes than in *in vitro*-matured oocytes. The zona pellucida of ovulated pig oocytes contains additional macromolecules (Hedrick *et al.*, 1987), such as the oestrogen-dependent oviductal glycoproteins

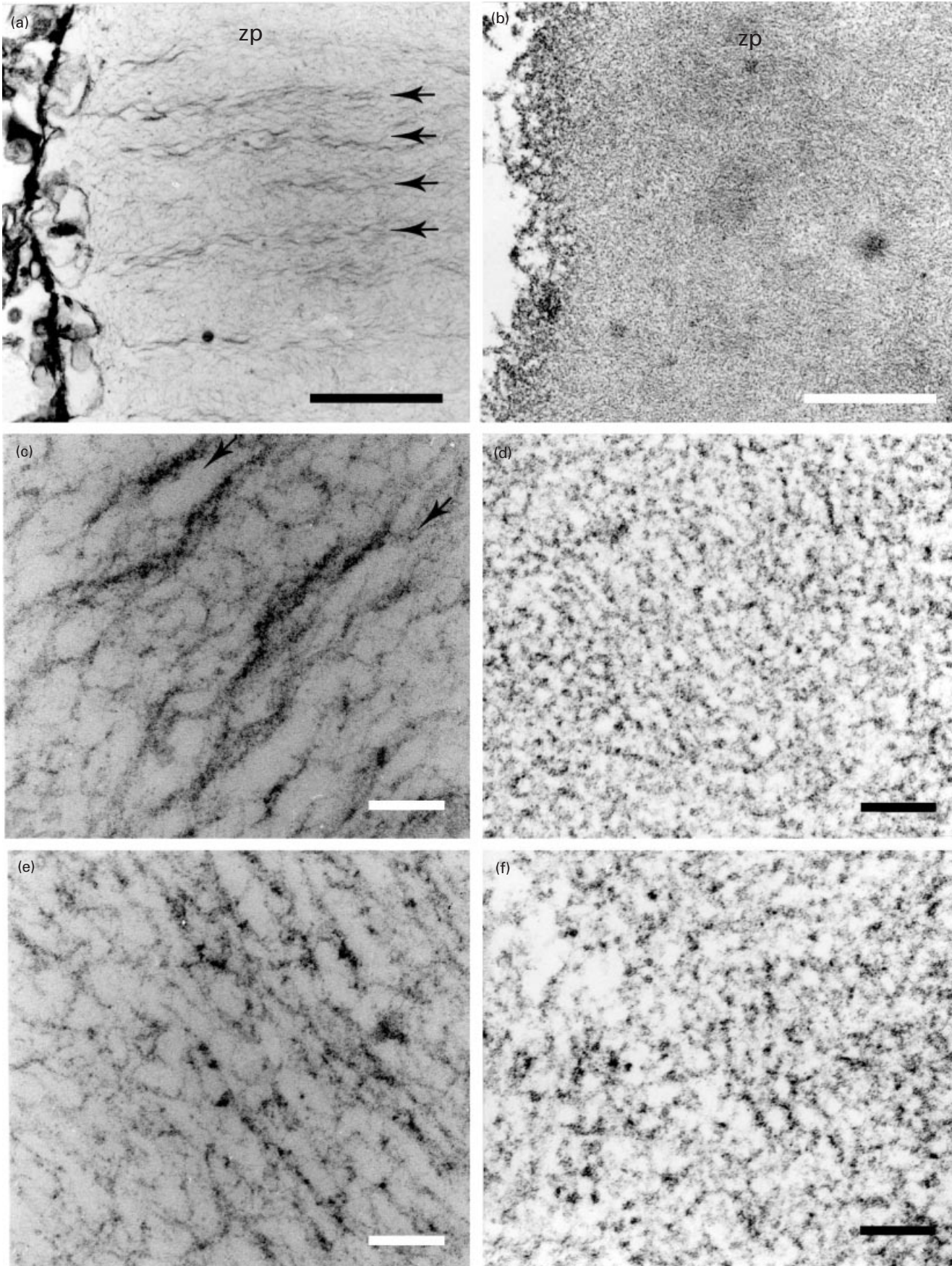
secreted into the oviductal lumen (Brown and Cheng, 1986; Buhi *et al.*, 1993). The zona pellucida of ovulated oocytes, but not of *in vitro*-matured oocytes, has resistance to dissolution by pronase (Wang *et al.*, 1998a), and it has been demonstrated that the resistance of *in vitro*-matured oocytes



**Fig. 5.** Transmission electron microscope images of the zonae pellucidae of an *in vivo* pig zygote. (a) A part of the zona pellucida (zp). o: ooplasm. (b) Outer part of zona pellucida. spz: sperm tail. (c) Inner part of zona pellucida. m: mitochondrion. (d) Outer part of zona pellucida. (e) Inner part of zona pellucida. Scale bars represent 1  $\mu\text{m}$  (a,b,c) or 100 nm (d,e).

increases after exposure to oviductal fluid (Kim *et al.*, 1996). As the resistance of ovulated oocytes was much higher in the outer surface of the zona pellucida (H. Funahashi, unpublished), the coarse fibrillar network observed in

ovulated oocytes may be the result of permeation of the oestrogen-dependent oviductal glycoproteins containing pronase inhibitors. However, aggregated fibrils in the inner zona pellucida were observed in both ovulated and *in vitro*-





matured oocytes, although the degree of aggregation of ovulated oocytes was much higher than in *in vitro*-matured oocytes. Therefore, the inner structure of the zona pellucida may be stiffer than the outer surface. Furthermore, as a large number of accessory spermatozoa can penetrate the outer portions of the zona pellucida only, it has been thought that materials from the cortical granules must be localized or initiate modifications in some specific way in the innermost portion of the zona pellucida (Hunter, 1991a). This different penetrability of spermatozoa between outer and inner zona pellucida may be explained by different zona pellucida structures; the coarse fibrillar network in the outer zona pellucida and aggregated fibrils in the inner zona pellucida. Moreover, the presence of channel-like, radially orientated fibrils in the *in vitro* zygotes, detected when a pre-fixation ruthenium red and saponin treatment was used, reinforces the idea that the cortical granules are not causing a major complete change in the inner zona pellucida, as may be the case in *in vivo* zygotes. This finding might reflect the inability of the zona pellucida of the *in vitro* zygotes to prevent the entry of accessory spermatozoa to such an extent as the *in vivo* zygotes. However, further research is required to determine why the degree of fibrillar aggregation of zona pellucida in ovulated oocytes is much higher than that in *in vitro*-matured oocytes.

Funahashi *et al.* (2000) demonstrated that the surface morphology of the zona pellucida in both ovulated and *in vitro*-matured oocytes changes after sperm penetration, and that both the outer and inner zona pellucida surfaces are lysed and lose the mesh-like structure during the zona pellucida reaction. In the present study, the external mesh-like structure was less apparent after sperm penetration, and fibrillar networks in both the outer and inner zona pellucida, particularly the outer network, were much more compacted after sperm penetration; however, collection of the *in vitro* zygotes 6–10 h later than their *in vivo* counterparts may affect the findings. These data demonstrate that the cross-sectioned morphology of zonae pellucidae in pig oocytes changes in both ovulated and *in vitro*-matured oocytes after sperm penetration *in vivo* and *in vitro*, respectively. However, the degree of morphological change in cross-sectioned zonae pellucidae during fertilization was much higher in ovulated oocytes than *in vitro*-matured oocytes. In a physiological situation, the defence mechanism against polyspermy in pig oocytes is achieved mainly by zona pellucida reaction after exocytosis of cortical granules against sperm penetration (Hunter, 1991b). However, Wang *et al.* (1998a) showed that the mean numbers of cortical granules beneath the plasma membrane before and after sperm penetration were not different between *in vitro*-matured and ovulated oocytes. In the present study, complete

cortical release was observed in *in vitro* zygotes. This evidence indicates that the differences in the morphology of cross-sectioned zonae pellucida during fertilization may be due to different zona pellucida morphology in ovulated and *in vitro*-matured oocytes, rather than to different amounts of cortical release. However, as induction of cortical granule exocytosis of *in vitro*-matured oocytes by calcium ionophore A23187 resulted in prevention of sperm penetration of the zona pellucida (Wang *et al.*, 1998b), even the subtle morphological changes observed in *in vitro* zygotes in the present study may be sufficient to prevent multiple sperm penetration. However, polyspermic penetration still remains the major obstacle to successful production of large numbers of pig embryos with high levels of developmental competence by *in vitro* maturation and fertilization (Funahashi and Day, 1997; Day, 2000). Therefore, a delayed or incomplete zona pellucida reaction may be one of the factors that permits an abnormally high incidence of polyspermic penetration of pig oocytes *in vitro*.

In the present study, the mean thickness of the zona pellucida was greater in ovulated oocytes than in *in vitro*-matured oocytes. The thickness of the zona pellucida of ovulated oocytes increased during fertilization, but did not change during IVF of *in vitro*-matured oocytes. These results are consistent with previous observations with SEM (Funahashi *et al.*, 2000), despite the different processing after fixation for SEM and TEM, even when ruthenium red and saponin pre-fixation was used. As the diameter is measured by using ultrathin sections with a maximum diameter of the specimen only, our data should reflect the mean thickness of the zona pellucida. Therefore, these observations demonstrate that the zona pellucida reaction of *in vitro* zygotes may be far from complete. In the present study, we could not clarify whether final follicular maturation occurred in follicles just before ovulation or in the oviduct after ovulation as the cross-sectioned zona pellucida structure was not compared between follicular oocytes matured *in vivo* (mature oocytes in dominant follicles just before ovulation) and ovulated oocytes. Further investigation may be required to clarify the final zona pellucida maturation.

In conclusion, TEM indicates that the fine structure of the zona pellucida and the zona pellucida reaction at sperm penetration differs between pig oocytes fertilized *in vivo* and *in vitro*, and that the outer and inner pig zonae pellucidae have different network organizations, probably due to insufficient final maturation of the zona pellucida during *in vitro* maturation. Exposure to oviductal glycoproteins or final follicular maturation may contribute to complete maturation and induce the penetration-dependent reaction of pig zonae pellucidae. Although

**Fig. 6.** Transmission electron microscope images of the zona pellucida (zp) of an *in vitro*-fertilized pig ovum (a,c,e) and *in vivo* zygote (b,d,f), prefixed with ruthenium red and saponin and fixed further with glutaraldehyde. (a,b) The inner area of the zona pellucida is different between the (a) *in vitro*-fertilized and (b) *in vivo* zygote, with radially-orientated fibrils in (a) (arrows), that are absent in the latter (b). The higher magnification of these areas in (c) and (d) shows the clear radial versus trabecule-like orientations. The outer part of the zona pellucida partially loses these orientations (e versus f). Scale bar represents 1  $\mu\text{m}$  (a,b) or 100 nm (c–f).

recent developments in pig *in vitro* maturation systems have made it possible to produce piglets efficiently from oocytes obtained at abattoirs, further investigation is needed to reproduce the zona pellucida morphology and reaction of ovulated oocytes.

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