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Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst *in vivo* and *in vitro*

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Summary

Lipid content in mammalian oocytes or embryos differs among species, with bovine and porcine oocytes and embryos showing large cytoplasmic droplets. These droplets are considered to play important roles in energy metabolism during oocyte maturation, fertilisation and early embryonic development, and also in the freezing ability of oocytes or embryos; however, their detailed distribution or function is not well understood. In the present study, changes in the distribution and morphology of porcine lipid droplets during *in vivo* and *in vitro* fertilisation, in contrast to parthenogenetic oocyte activation, as well as during their development to blastocyst stage, were evaluated by transmission electron microscopy (TEM). The analysis of semi-thin and ultra-thin sections by TEM showed conspicuous, large, electron-dense lipid droplets, sometimes associated with mitochondrial aggregates in the oocytes, irrespective of whether the oocytes had been matured *in vivo* or *in vitro*. Immediately after sperm penetration, the electron density of the lipid droplets was lost in both the *in vivo* and *in vitro* oocytes, the reduction being most evident in the oocytes developed *in vitro*. Density was restored in the proncular oocytes, fully in the *in vivo* specimens but only partially in the *in vitro* ones. The number and size of the droplets seemed, however, to have decreased. At 2- to 4-cell and blastocyst stages, the features of the lipid droplets were almost the same as those of pronuclear oocytes, showing a homogeneous or saturated density in the *in vivo* embryos but a marbled or partially saturated appearance in the *in vitro* embryos. *In vitro* matured oocytes undergoing parthenogenesis had lipid droplets that resembled those of fertilised oocytes until the pronuclear stage. Overall, results indicate variations in both the morphology and amount of cytoplasmic lipid droplets during porcine oocyte maturation, fertilisation and early embryo development as well as differences between *in vivo* and *in vitro* development, suggesting both different energy status during preimplantation development in pigs and substantial differences between *in vitro* and *in vivo* development.

Keywords: Culture, *In vitro*, *In vivo*, Lipid droplet, Pig

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Introduction

Lipid droplet content in mammalian oocytes or embryos differs among species. Bovine and porcine oocytes and embryos usually show large amounts of lipid droplets, in contrast to rodent or human oocytes. Lipid droplets are considered to be a source of energy (Brown, 2001) and seem to play important roles in oocyte maturation, fertilisation and development to embryos. Lipid content rates, furthermore, to higher sensitivity to freezing during cryopreservation of embryos in cows (Mohr & Trounson, 1981; Leibo & Loskutoff, 1993; Yamashita *et al*., 1999; Abe *et al*., 2002)

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and pigs (Nagashima *et al.*, 1995). Although freezing of porcine embryos derived *in vivo* has been successful (reviewed by Dobrinsky, 2002), there are no reports describing successful cryopreservation of *in vitro* produced embryos. Although the viability of *in vitro* embryos is considered to be lower than that of *in vivo* ones, it is possible that a difference in their lipid contents results in a different sensitivity to freezing. However, little is known about lipid droplet distribution or function throughout maturational and developmental stages, or about the difference between *in vivo* and *in vitro* materials.

It is possible to detect cytoplasmic lipids. Methods have been established to measure the content of lipid or fatty acids in bovine oocytes or embryos (Ferguson & Leese, 1999; Sata *et al.*, 1999; McEvoy *et al.*, 2000; Kim *et al.*, 2001). For example, the lipid content and fatty acid composition of the total lipid fraction has been analysed by gas chromatography in matured oocytes (Kim *et al.*, 2001). On the other hand, morphological evaluation of lipid droplet distribution seems to be a rather simple method to determine their content and follow changes in localisation during development. Lipid deposits often accumulate in large masses, being defined as 'lipid droplets', which can be easily observed in sections. Use of transmission electron microscopy (TEM) procedures reveals droplets of high electron density following fixation with osmium tetroxide. Ultrastructural analyses of bovine (Plante & King, 1994; Abe *et al.*, 1999*a*, *b*; Crosier *et al.*, 2001) and porcine (Hyttel & Niemann, 1990) embryos up to the blastocyst stage have been performed. However, detailed information about transient changes in lipid droplets during embryo development is rather scarce (bovine: Abe *et al.*, 1999*a*, *b*). The present study was undertaken to determine the morphological changes in lipid droplets during porcine fertilisation, parthenogenetic activation and early embryonic development *in vivo* and *in vitro*, using sections evaluated by light microscopy and TEM.

Materials and methods

In vivo-developed gametes, zygotes and embryos

All *in vivo*-developed gametes, zygotes and embryos were collected from normally cycling multiparous cross-bred (Swedish Yorkshire × Swedish Landrace) sows, recruited for the experiments after weaning, and individually penned at the Department of Obstetrics and Gynaecology, SLU, Uppsala. The sows received standard ration and water *ad libitum*, according to Swedish standards (Simonsson, 1994). Fertile boars were always penned in the vicinity. The sows were checked twice daily by experienced personnel for behavioural oestrus. Six sows (out of 8) were randomly mated twice (at 12 h and 24 after onset of oestrus) with one of two fertile boars. Occurrence of ovulation was recorded by transrectal ultrasonography (TUS) using an annular array scanner (Scanner 250, Pie Medical, Maastricht, The Netherlands) with a 5 MHz multiple-angle transducer. A specially constructed wagon was used to immobilise the sows during TUS scanning (Mburu *et al.*, 1995). The experimental design was reviewed and approved by the Ethics Committee for Experimentation with Animals in Sweden.

Follicular oocytes were collected from two sows that showed onset of oestrus about 36 h prior to slaughter. After denudation of cumulus cells by hyaluronidase treatment and gentle pipetting, oocytes with the first polar body were categorised as *in vivo*-matured oocytes. Ovulated oocytes were collected at approximately 5 h after ovulation from a mated sow. These oocytes were categorised as *in vivo*-fertilised oocytes. At 10 h after the confirmation of ovulation, oviductal oocytes were also collected from another two mated sows. They were categorised as *in vivo* pronuclear oocytes. Some of the oocytes from these groups were fixed and evaluated for nuclear status. On the second day after ovulation, cleaved embryos were collected as *in vivo* early embryos from a mated sow, and on the sixth day, blastocysts were also collected as *in vivo* blastocysts from the other two sows. Five to 12 oocytes or embryos were collected from each sow.

In vitro maturation (IVM), fertilisation (IVF) and culture (IVC)

All the *in vitro* materials were obtained as described previously (Kikuchi *et al.*, 1999*a*, 2002). In brief, porcine ovaries from pre-pubertal Large White gilts were obtained at a local slaughterhouse and transported to the laboratory at 35 °C. Cumulus–oocyte complexes (COCs) were collected from follicles 3–5 mm in diameter in a collection medium that consisted of Medium 199 (with Hanks' salts; Gibco, Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium (Sigma Chemical, St Louis, MO) and 0.1 mg/ml streptomycin sulfate (Sigma) (Kikuchi *et al.*, 1993). About 30 COCs were cultured in each 500 μl of maturation medium, a modified NCSU-37 solution (Petters & Wells, 1993; Funahashi *et al.*, 1997) containing 10% (v/v) porcine follicular fluid, 50 μM β-mercaptoethanol, 0.6 M cysteine, 1 M dibutytyl cAMP (dbcAMP, Sigma), 10 IU/ml eCG (PMS 1000 IU, Nihon Zenyaku Kogyo, Koriyama, Japan) and 10 IU/ml hCG (Puberogen 500 unit, Sankyo, Tokyo, Japan), in 4-well dishes (Nunclon Multidishes, Nalge Nunc International, Denmark) for 20–22 h. They were subsequently cultured in the maturation medium without

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dbcAMP and hormones for 24 h. The maturation culture was carried out under conditions of O_2 CO_2 and N_2 adjusted to 5%, 5% and 90%, respectively, at 39 °C.

After denudation, oocytes with the first polar body were collected as *in vitro*-matured oocytes. Frozen and thawed epididymal spermatozoa (Kikuchi *et al*., 1998) from a Landrace boar were preincubated for 1 h at 37 °C in Medium 199 adjusted to pH 7.8 (Nagai *et al*., 1988). Fertilisation medium for porcine oocytes (Pig-FM) (Suzuki *et al*., 2000) consisting of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate and 10 mM HEPES was modified by Suzuki (Suzuki *et al*., 2002) by the addition of 8 mM $CaCl_2$, 2 mM sodium pyruvate, 2 mM caffeine and 5 mg/ml bovine serum albumin (BSA; Fraction V, Sigma). A portion (10 µl) of the preincubated spermatozoa was introduced into 90 µl fertilisation medium containing about 20 COCs surrounded by expanded cumulus cells. The final sperm concentration was adjusted to 1×10^5 cells/ml. Co-incubation was carried out for 3 h at 39 °C under 5% O_2. Then the oocytes were freed from the cumulus cells and attached spermatozoa.

Some oocytes were collected as *in vitro*-fertilised oocytes. The others were transferred into IVC-PyrLac medium (NCSU-37 medium without glucose, but containing 4 mg/ml BSA, 50 µM β-mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium lactate), conditioned by the oviductal epithelial cells (Kikuchi *et al*., 2002), and subsequently cultured *in vitro*. Some inseminated oocytes at 10 h post-insemination and the 3- to 4-cell stage embryos on the second day (day 2) were collected as *in vitro* pronuclear oocytes and *in vitro* early embryos, respectively. On day 2, the remaining embryos were transferred to IVC-Glu medium (NCSU-37 plus BSA and β-mercaptoethanol) and cultured for a further 4 days (until day 6). At day 6, expanded blastocyst were harvested as *in vitro* blastocysts. The collections were carried out twice. A total of 70 oocytes or embryos for each category were harvested for evaluation by light microscopy and TEM.

Parthenogenetic activation of IVM oocytes

In vitro-matured oocytes were selected and stimulated as previously described (Kikuchi *et al*., 1995). They were transferred to activation solution consisting of 0.28 mM *d*-mannitol (Wako Pure Chemical, Tokyo, Japan), 0.05 mM $CaCl_2 2H_2O$ (Wako), 0.1 mM $MgCl_2 2H_2O$ (Wako) and 0.01 mg/ml BSA. They were transferred to the hybridising chamber (FTC-22W, Shimadzu, Tokyo, Japan) containing 50 µl activation solution and stimulated with a 20 µs pulse at 1.5 kV/cm DC using a somatic hybridiser (SSH-10, Shimadzu). The stimulated oocytes were subsequently cultured for 3 h, 10 h and 20 h and harvested as parthenogenetic stimulated oocytes (3 h) and parthenogenetic pronuclear oocytes (10 and 20 h), respectively. The collections were carried out twice. A total of 70 oocytes or embryos for each category were harvested for evaluation by light microscopy and TEM.

Evaluation of oocyte/embryo stage under light microscopy

For the confirmation of oocyte/embryo nuclear stage, some of the harvested oocytes or embryos (a total of 3–5 for *in vivo* materials and about 50 for *in vitro* materials in each category) were whole-mounted and fixed in acetic alcohol (1:3). After staining with 1% (w/v) aceto-orcein solution, they were evaluated by a light microscope equipped with phase-contrast optics.

Evaluation by TEM

All the remaining oocytes and embryos were fixed in a 2.5% solution of glutaraldehyde in sodium cacodylate buffer (0.067 M, pH 7.2–7.4) and stored in the same solution at 4 °C until being processed. After post-fixation for 5 min in 2% (w/v) osmium tetroxide in the cacodylate buffer, specimens were embedded in Agar^{100} resin (Agar Scientific, Cambridge, UK). Semi-thin section and continuous ultra-thin sections of oocytes and embryos were processed conventionally for TEM. Semi-thin sections for light microscopy were stained with toluidine blue. Thereafter, ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and examined by a TEM (Philips 420 electron microscope, Einhoven, The Netherlands) at 80 kV. Five to seven oocytes or embryos for each category were evaluated.

Results

Developmental stages of all the categorised oocytes or embryos in the present study were confirmed with whole-mounted specimen (Table 1). The stages of the *in vivo* materials were almost uniform and synchronised in each collection. *In vivo*-fertilised oocytes were estimated to be at 3–6 h after gamete encounter since they were found to be at telophase II with the identified second polar body or at an early stage of pronucleus formulation. *In vivo* pronuclear oocytes were estimated to be at 4–20 h after gamete encounter due to the existence of well-developed male and female pronuclei. Two-cell stage embryos and hatched blastocysts were collected as *in vivo* early embryos and *in vivo* blastocyts, respectively. On the other hand, *in vitro* specimens were not at the same stage because all the oocytes were not matured and fertilised. In the present study, about 70% (37/53) of the cultured oocytes were matured and about 50% (18/37) of the matured oocytes

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Table 1 Developmental stages of oocytes or embryos as categorised in Materials and Methods

Oocyte/ embryo	Categorised as: Matured oocyte	Fertilised or activated[a] oocyte	Pronuclear oocyte	Early embryo	Blastocyst
In vivo	Metaphase II	Telophase II or early pronuclear stage	FPN and MPN formed	2-cell storage	Hatched blastocysts
In vitro	Metaphase II	Anaphase II or telophase II	FPN and MPN formed	3- to 4-cell stage	Expanded blastocysts
Parthenogenetic[a]	–	Telophase II	FPN formed or fragmented oocytes[b]	–	–

FPN, female pronucleus; MPN, male pronucleus.
[a]*In vitro*-matured oocytes were stimulated with an electric pulse.
[b]Some fragmented oocytes were seen at 20 h after stimulation. In the present study, pronuclear oocytes at 10 and 20 h were collected.

were fertilised, where the monosperm fertilisation rate was about 30% (11/37). Most fertilised oocytes were at the anaphase II or telophage II stage at 3 h after insemination, and they formed both male and female pronuclei at 10 h (average number of penetrated spermatozoa was 3.2). We carefully checked for evidence of fertilisation – for example, extrusion of cortical granules, existence of the second polar body or pronucleus – during preparation of semi-thin sections for TEM. On day 2 of IVC, the percentage of 3- to 4-cell stage embryos was 42% (20/48). They were collected as *in vitro* early embryos. On day 6, the blastocyst formation rate was 20% (10/51). The expanded blastocysts were collected as *in vitro* blastocysts. As regards parthenogenesis, some of the stimulated oocytes were activated to telophase II stage at 3 h after the stimulation, and more than 80% (42/50) of the oocytes formed female pronuclei at 10 h. Some fragmented oocytes existed but most of them were pronuclear oocytes even at 20 h.

Semi-thin sections from matured oocytes, fertilised oocytes and embryos, and also parthenogenetic activated oocytes, are shown in Figs. 1–3, and ultra-thin sections in Figs. 4–6. The analysis of ultra-thin sections at TEM showed conspicuous, large, electron-dense lipid droplets, sometimes associated with mitochondrial aggregates in the oocytes, irrespective of these being matured *in vivo* or *in vitro* (Fig. 4*a* and *d*, respectively). Lipid droplets in matured oocytes featured finely marbled or partially saturated figures, with an associated homogeneous area. The electron density of the lipid droplets was lost in both the *in vivo* and *in vitro* oocytes immediately after sperm penetration and the saturated structure was barely seen in either oocyte type (Fig. 4*b* and *e*, respectively). The density of the lipid droplets in the *in vivo*-fertilised oocytes was reduced to pale grey, while the density seemed to be almost completely lost in the *in vitro*-fertilised oocytes. At the pronculear stage, electron density was restored fully in the *in vivo* specimens but only partially in the *in vitro* ones (Fig. 4*c* and *f*, respectively). The structure in the *in vivo* droplets always appeared homogeneous in density, while that in the *in vitro* ones showed a distinct marbled or partially saturated appearance. These changes in lipid density were clearly observed in semi-thin sections as well (Fig. 1*a*–*f*).

During embryonic development, the electron density of the lipid droplets was high in both the *in vivo* and the *in vitro* embryos, whereas the number and size of the droplets in the *in vitro* specimens were larger than in those *in vivo* (Fig. 2*a*–*d*). The fine structure confirmed these observations (Fig. 5*a*–*d*). The droplets in the *in vivo* embryos were homogeneous with a higher electron density than the *in vitro* specimens. On the other hand, the droplets in the *in vitro* early embryos had similar features to those in the *in vivo*-matured or pronuclear oocytes. The *in vitro*-developed blastocysts had lipid droplets with a higher electron density than in the *in vivo* ones, albeit with a lower homogeneity. These features were observed in both inner cell mass and trophoblast cells.

When *in vitro*-matured oocytes were stimulated with electric pulses, the density of the droplets in both semi-thin and ultra-thin sections was not reduced by 3 h after the stimulation (Figs. 3*a* and 6*a*, respectively). This feature was similar to that in the pronuclear oocytes both *in vivo* and *in vitro*, and this morphological appearance was almost the same until 10 or 20 h after stimulation in both semi-thin (Fig. 3*b* and *c*, respectively) and ultra-thin (Fig. 6*b* and *c*, respectively) sections.

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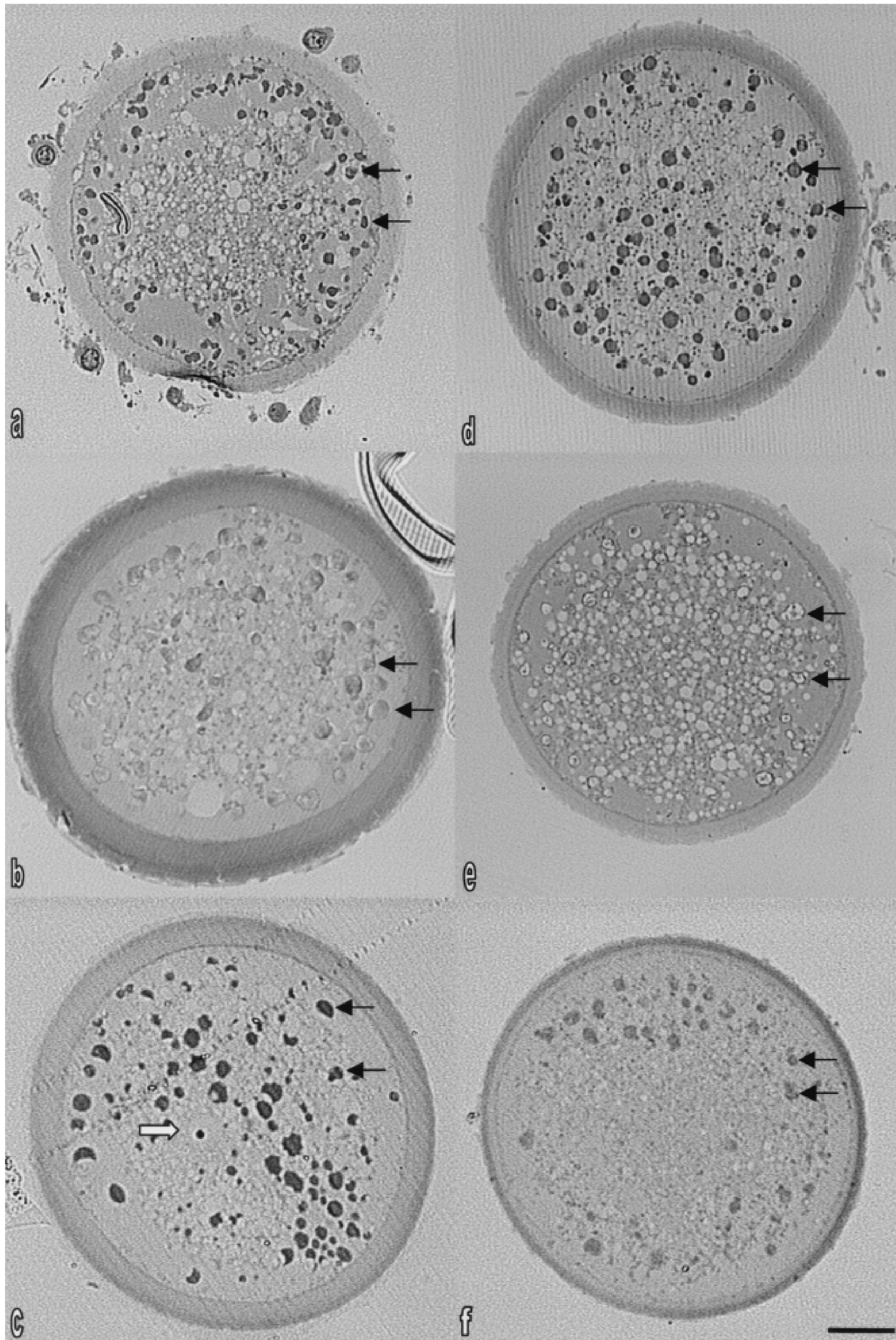

Figure 1 Light micrographs of semi-thin sections of porcine oocytes *in vivo* (*a*–*c*) or *in vitro* (*d*–*f*) matured (*a*, *d*), fertilised (*b*, *e*) or at the pronuclear stage (*c*, *f*) depicting lipid droplet (small arrows) localised throughout. Note the lower density of the lipid droplets at fertilisation compared with matured oocytes, and the restoration of the higher density at the proncuclear stage. White arrow, pronucleus. Scale bar represents 20 μm.

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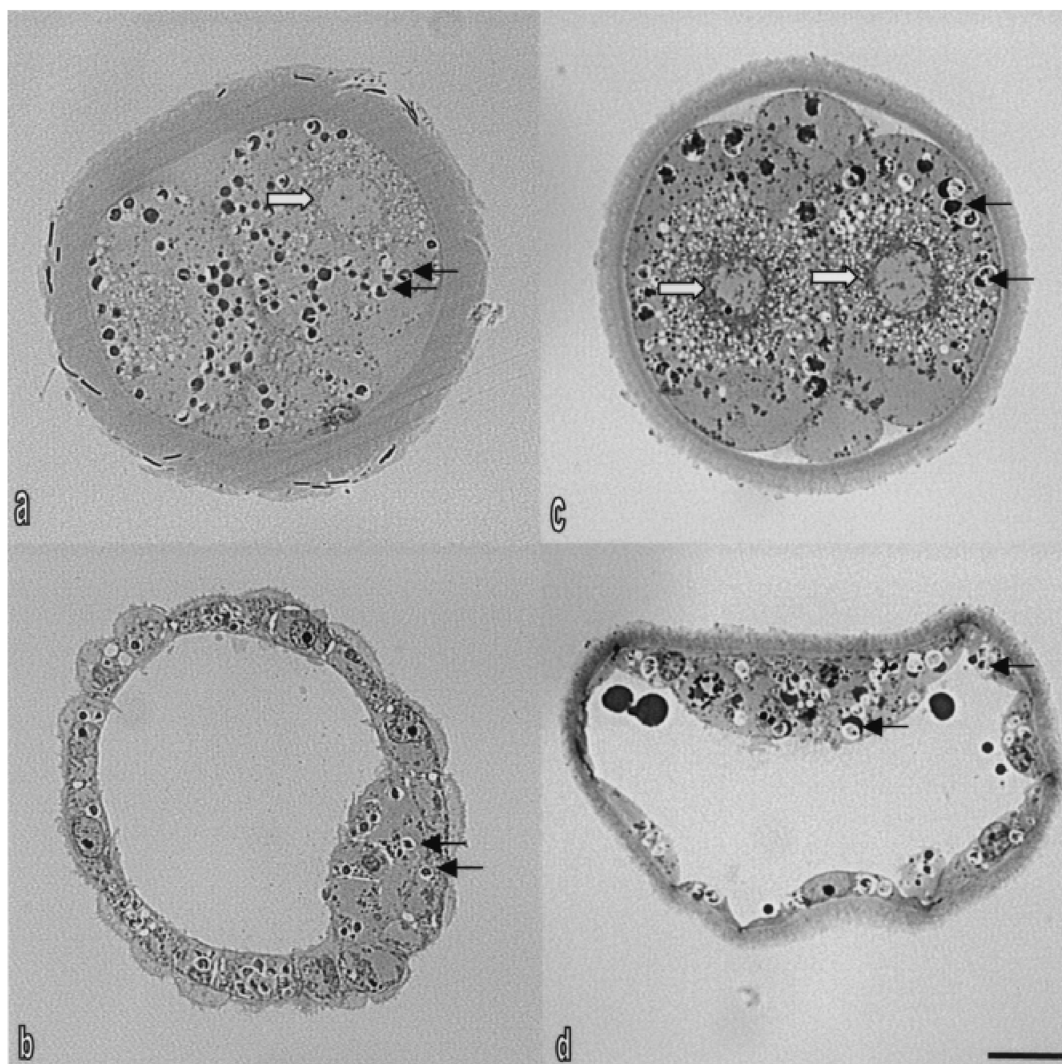

Figure 2 Light micrographs of semi-thin sections of porcine early embryos 2 days (*a*, *c*) and 5–6 days (*b*, *d*) post-fertilisation, developed *in vivo* (*a*, *b*) or *in vitro* (*c*, *d*), showing the localisation of lipid droplets (small arrows) in the blastomeres. Note the more homogeneous, dense aspect of lipid deposited in the *in vivo*-developed embryos compared with the larger droplets in the *in vitro* embryos. White arrows, nucleus. Scale bar represents 20 μm.

Discussion

The mechanisms by which lipid droplets develop or degenerate are not well understood. During the development of lipid droplets in adipocytes, it is reported that a number of proteins, such as perilipins (perilipin A, B and C) or caveolin/oleosin, play an important role in the formation of the droplets as lipid deposits (Brown, 2001). However, the mechanism behind the degradation of lipid droplets has not been reported in any cell, including oocytes or embryos. Lipid droplets serve as storage depots for neutral lipids such as triglyceride and sterol esters (Brown, 2001). It may be possible that the morphological changes in lipid droplets observed during fertilisation and early embryonic development depend on the energy status in these cell types, because the density of the droplets after osmium fixation varies depending on the degree of saturation of triglycerides (Abe *et al.*, 1999*a*). In the present study, the density of the lipid droplets in the oocytes matured both *in vivo* and *in vitro* decreased just after fertilisation and was restored again at the pronuclear stage. These results emphasise that energy consumption is accelerated for the completion of the early stage of fertilisation, where numerous nuclear and cytoplasmic changes occur such as membrane fusion with the sperm surface, cortical granule reaction, replacement of sperm nuclear protamines (Shimada *et al.*, 2000) by histones (Nakazawa *et al.*, 2002), sperm head decondensation, pronuclear membrane formation, replication of gamete DNA, etc. Energy substrates for the completion of these changes

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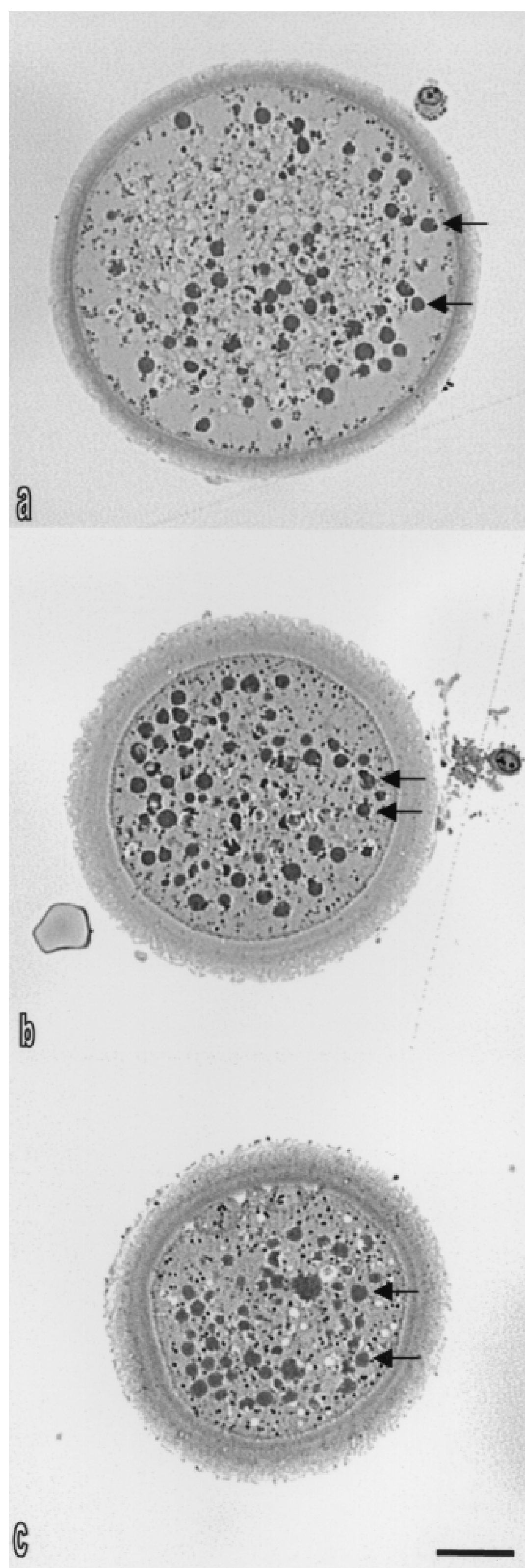

Figure 3 Light micrographs of semi-thin sections of porcine *in vitro*-matured oocytes submitted to electrostimulation and fixed for TEM at 3 h (*a*), 10 h (*b*) and 20 h (*c*) thereafter. Lipid droplets (small arrows) have the same appearance throughout. Scale bar represents 20 µm.

may be related to changes in cytoplasmic lipid storage. It has been reported that, in toad eggs, fertilisation triggers a decrease in triglycerides and diglycerides while, in contrast, free fatty acid increases continuously (Alonso *et al.*, 1986). This report suggests that the decrease in triglycerides is a reflection of the activation of lipolytic enzymes and the subsequent oxidation of fatty acids to meet the increasing metabolic energy requirements brought on by external fertilisation. Although the report was not from mammalian species, it is possible that mammalian oocytes also undergo such dramatic changes during internal fertilisation. After the storage of cytoplasmic lipids for fertilisation, morphological and functional changes may occur in the appearance and distribution of lipid droplets. At the pronuclear stage, enough energy is supplied from the droplets to accomplish the events of fertilisation; on the other hand, lipid droplet storage may be restored again by the development of new lipid droplets.

In contrast to the fertilised oocytes, it is interesting that the oocytes stimulated parthenogenetically did not show any such dramatic change. The reason is yet to be clarified. Oocyte activation seems to differ between fertilisation and parthenogenesis. From the point of view of the cell cycle, both fertilisation (Kikuchi *et al.*, 1999*b*) and parthenogenesis (Kikuchi *et al.*, 1995) induce a common cytoplasmic change, an inactivation of M-phase promoting factor, which results in nuclear progression from M-phase to interphase. However, other cytoplasmic aspects do not seem to be completed in parthenogenesis. For example, the cortical reaction is affected by the activation method (Sun *et al.*, 2001), and electric pulses can induce nuclear activation but not the cortical reaction in some species (Gulyas, 1980). In the present study, electric pulses were used to induce nuclear activation, resulting in more than 80% of stimulated oocytes forming a female pronucleus. However, most of the activated oocytes still contained a relatively large amount of cortical granules even at 10 h after the stimulation when they were evaluated by both semi-thin (Fig. 3*b*) and ultra-thin (Fig. 6*b*) sections. The method of parthenogenetic stimulation used in the present does not seem to induce full cytoplasmic activation, resulting in the consumption of only a small amount of the oocyte's energy source, and this may explain why the morphology of the lipid droplets did not change dramatically even after nuclear activation.

It is reported that the cytoplasm of bovine embryos produced *in vivo* or *in vitro* contains numerous lipid droplets prior to the blastocyst stage (Plante & King, 1994; Abe *et al.*, 2002) and, as bovine embryos develop, excess lipid may be sequestered within the cell and utilised by mitochondria for the increased production of ATP (Stojkovic *et al.*, 2001) required for blastocoele

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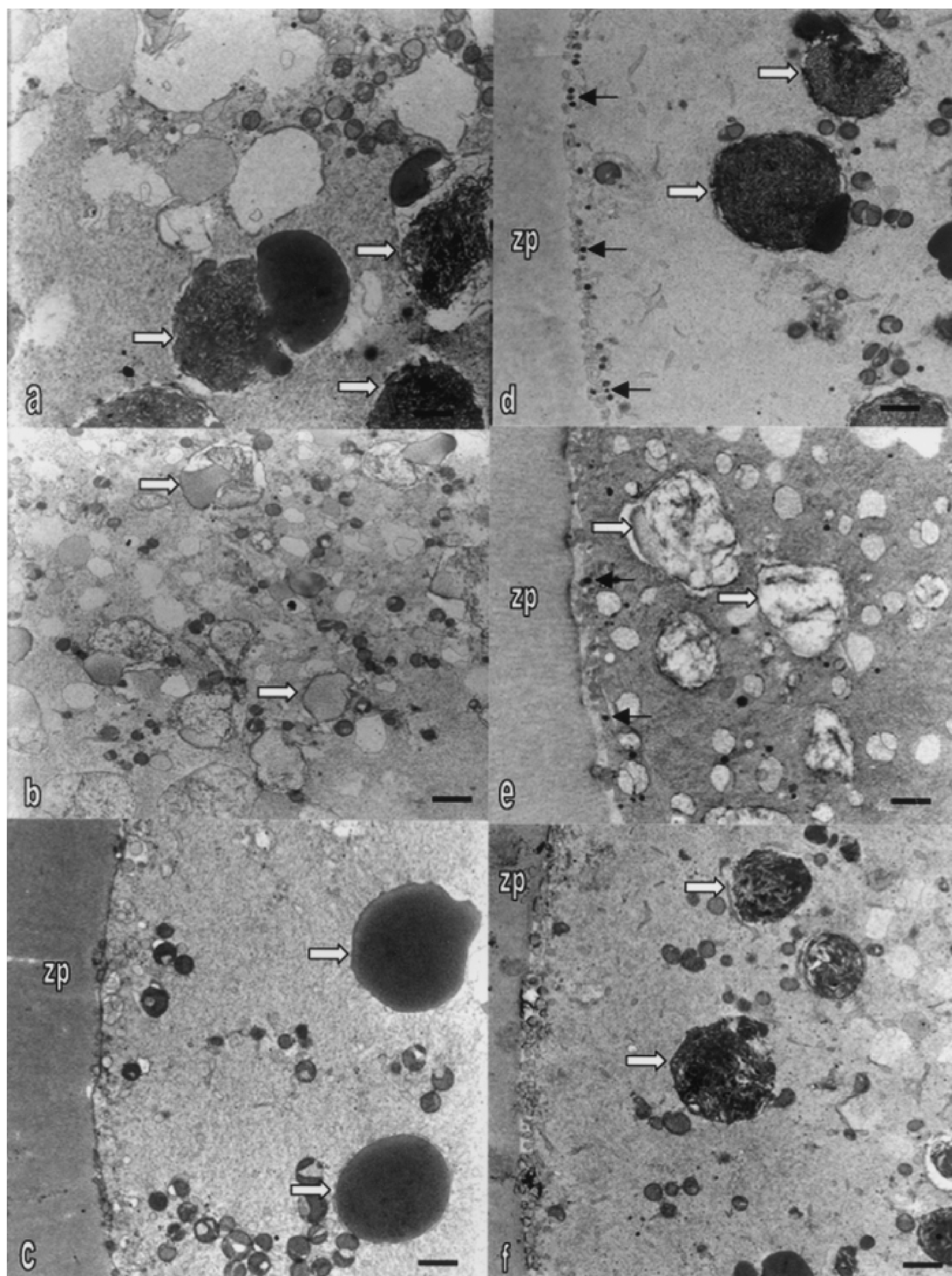

Figure 4 Transmission electron (TEM) micrographs of the cortical cytoplasm of porcine oocytes *in vivo* (*a*–*c*) or *in vitro* (*d*–*f*) matured (*a*, *d*) fertilised (*b*, *e*) or in pronuclear stage (*c*, *f*) depicting the appearance of the lipid droplets (white arrows). Note the more homogeneous, electron-dense appearance of the lipid droplets in the *in vivo*-developed oocytes. zp, zona pellucida; small arrows, cortical granules. Scale bar represents 5 μm.

formation or differentiation of cell lineages (Sathananthan & Trounson, 2000; Crosier *et al.*, 2001). However, a certain amount of lipid droplets still exist at the blastocyst stage in bovine (Abe *et al.*, 1999*a*, *b*, 2002; Crosier *et al.*, 2001) and also porcine (in the present study) embryos. Although lipid droplets were present in the blastocyst stage in both species, their morphological characteristics seem to be different. In

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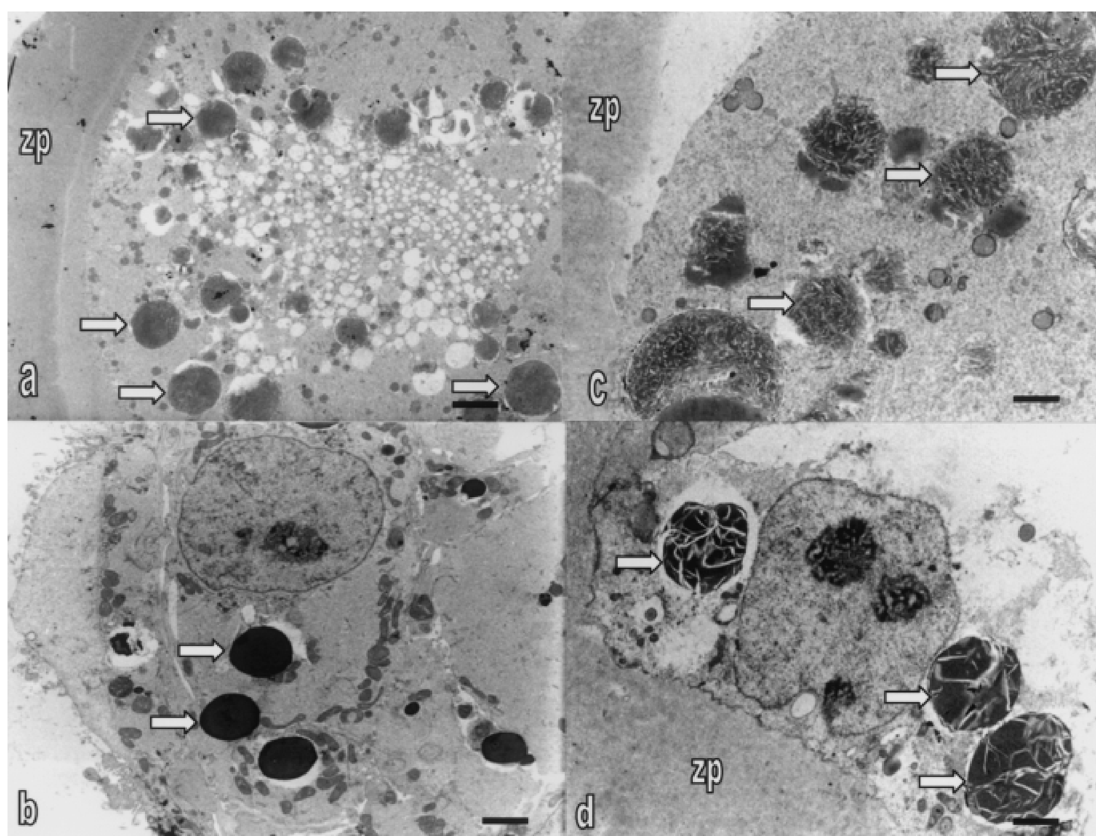

Figure 5 Transmission electron micrographs of porcine early embryos 2 days (*a*, *c*) and 5–6 days (*b*, *d*) post-fertilisation, developed *in vivo* (*a*, *b*) or *in vitro* (*c*, *d*), depicting the appearance of the lipid droplets (white arrows) in the blastomeres. Note the more homogeneous, electron-dense appearance of the lipid deposits in the *in vivo*-developed embryos, as seen previously in the oocytes. zp, zona pellucida. Scale bar represents 5 μm (*a*, *b*) or 10 μm (*c*, *d*).

bovine morulae and blastocysts, lipid droplets are homogeneous or fully saturated, showing a similar electron density in *in vivo*- or *in vitro*-produced embryos (Crosier *et al.*, 2001). Sometimes, lipid droplets have been partially saturated in appearance in both *in vivo* and *in vitro* embryos, and associated with lysosome-like vesicles (Mohr & Trounson, 1981; Abe *et al.*, 1999*a*, *b*). In addition, the number of lipid droplets in the *in vitro* blastocysts, cultured in a serum-containing medium, is higher than that in the *in vivo*-developed embryos (Abe *et al.*, 1999*a*, *b*). In contrast, in porcine embryos, the electron density of the lipid droplets present in *in vivo* embryos was homogeneous or fully saturated, while lipid droplets in *in vitro*-produced embryos appeared partially saturated (Fig. 4*a*–*d*). Lysosome-like vesicles were not apparent in either type of porcine specimen. These changes in the appearance of the lipid droplets in porcine embryos began to be observed at the pronuclear stage, and became fully apparent by the 2- to 4-cell and blastocyst stages, suggesting that the density of lipid droplets seems to be fully restored within the homogeneous appearance in the *in vivo* specimens but becomes only partially saturated in the *in vitro* specimens. The functional aspect of lipid saturation, which also was observed in a proportion of the droplets seen in matured oocytes, is not well understood, although the differences seen by TEM between *in vivo* and *in vitro* specimens are quite interesting. In bovine morulae or blastocysts, the homogeneity or saturated appearance of lipid droplets increases when they are cultured in serum-supplemented medium (Abe *et al.*, 1999*a*, *b*, 2002) suggesting the incorporation of triglyceride (Ferguson & Leese, 1999) or lipoprotein (Sata *et al.*, 1999) from serum. Our IVC medium for porcine zygotes is supplemented with BSA (Fraction V), which can be combined with fatty acid. This may cause an incomplete uptake of apoplipoprotein (the carrier protein for neutral lipid, phospholipid or cholesterol) (Abe *et al.*, 2000), due to the absence of serum in the porcine culture medium. In addition, lysosome-like vesicles are observed less frequently, especially in bovine blastocysts cultured in serum-supplemented medium, leaving a question mark as regards their function in relation to lipid deposits (Abe *et al.*, 1999*a*, *b*). Lysosome-like vesicles were not observed in either *in vivo* or *in vitro* porcine embryos.

The IVC system used in the present study has

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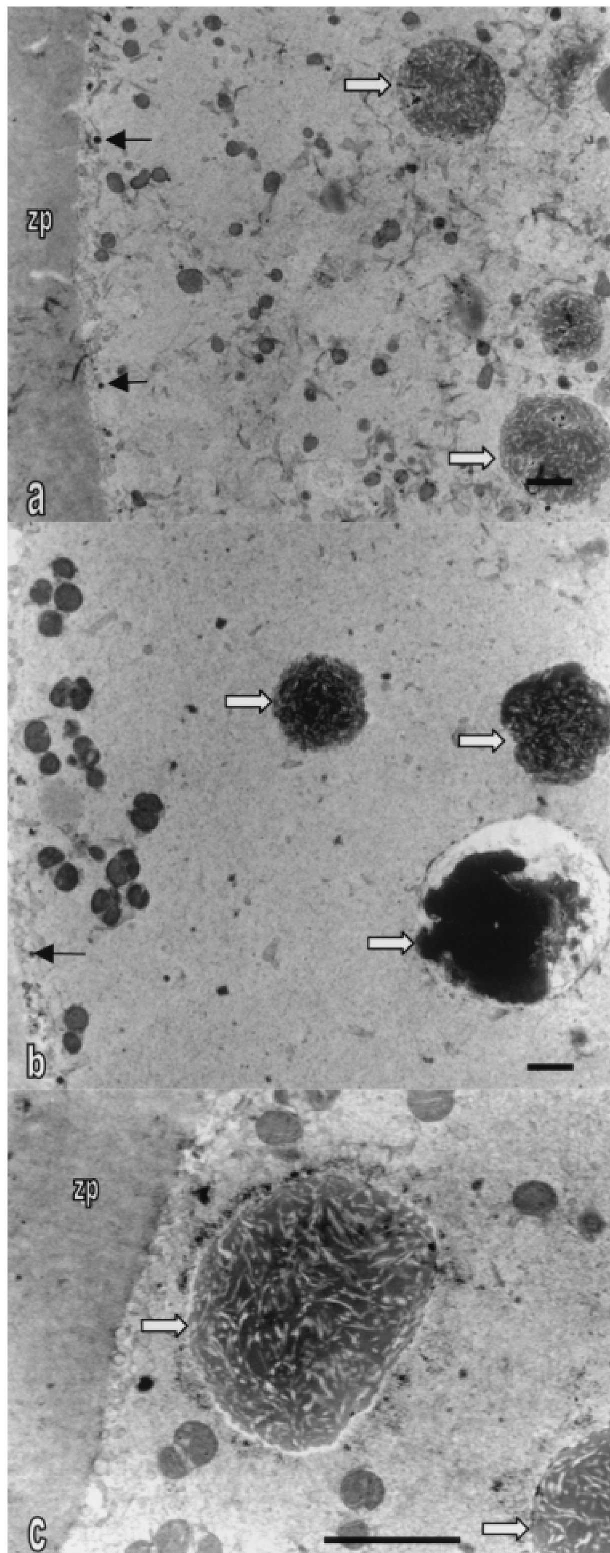

Figure 6 Transmission electron micrographs of the cortical cytoplasm of porcine *in vitro*-matured oocytes submitted to electrostimulation and fixed for TEM at 3 h (*a*), 10 h (*b*) and 20 h (*c*) thereafter. Note the appearance of the lipid droplets (white arrows) and the presence of cortical granules (small arrows) up to 10 h after electrostimulation. zp, zona pellucida. Scale bar represents 5 μm.

proved to have an advanced ability for *in vitro* blastocyst formation (average number of total cells of expanded blastocysts on day 6 = 86). Following transfer to recipients, the blastocysts developed to piglets (Kikuchi *et al.*, 2002). The present results suggest that there is a different morphology (perhaps reflected in a different function) of the lipid deposits in *in vitro* porcine embryos compared with *in vivo* embryos. In bovine embryos, an excess accumulation of lipid droplets is considered to be abnormal when they are cultured in serum-supplemented media (Abe *et al.*, 1999*a*), which relates to the viability of frozen and thawed embryos (Yamashita *et al.*, 1999; Abe *et al.*, 2002). However, considering that the *in vivo* porcine embryos have a more homogeneous content of lipid droplets, the accumulation of these lipid droplets seems to be normal for further embryonic development in pigs.

In conclusion, the present study indicates marked variations in the morphology and amount of cytoplasmic lipid droplets during porcine oocyte maturation and fertilisation, as well as in preimplantation embryos both *in vivo* and *in vitro*, perhaps in relation to differences in energy status during preimplantation development in pigs. Understanding these steps of lipid transition may provide clues for the optimisation of culture conditions or cryopreservation of porcine oocytes and/or embryos in this species.

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