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Effect of oviductal and cumulus cells on zona pellucida and cortical granules of porcine oocytes fertilized in vitro with epididymal spermatozoa

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Abstract

The objective of this study was to evaluate the effects of porcine oviductal epithelial cell (POEC) monolayers and cumulus cells on the zona pellucida (ZP) and cortical granules (CG) of in vitro matured porcine oocytes. Denuded and cumulus-enclosed oocytes were exposed to POEC before or during in vitro fertilization (IVF). The functional effects of the co-culture system were the tested on the ZP resistance, measured by the time necessary to dissolve the ZP with 0.1% pronase, and the distribution and density of the cortical granules. CG density in the equator and cortex of each oocyte was evaluated by confocal microscopy after staining with fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA). Both variables were assessed immediately after an in vitro maturation period (IVM group), 3 and 6 h after culture with or without (Control) oviductal cells (Experiment 1) and 3 h after insemination with frozen-thawed epididymal spermatozoa in the presence or absence (Control) of oviductal cells (Experiment 2). The time to dissolve the ZP of oocytes from IVM group was 440.4 ± 61.7 s and no difference was observed among groups in Experiment 1. In contrast, the density of CG was affected; oocytes pre-incubated for 6 h had a higher density than those pre-incubated for 3 h ($P < 0.001$). Oocytes fertilized in vitro in the presence of POEC (Experiment 2) had a similar ZP digestion time as control oocytes 3 h after insemination. The presence of POEC during IVF as well as the presence of cumulus cells had no effect on the density and distribution of CG. However, a significant decrease in the density of CG was observed in the fertilized oocytes compared to in vitro matured oocytes ($P < 0.001$). It is concluded that under the conditions employed the oviductal and cumulus cells in the perifertilization period had no effect on ZP hardening and CG density. However, an increase in CG density was observed when oocytes were maintained in culture.

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In addition, no hardening of ZP was observed after IVF, and denuded and cumulus-enclosed oocytes showed similar cortical reactions after insemination with epididymal spermatozoa regardless of the presence of POEC.
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1. Introduction

Prior to fertilization, the zona pellucida (ZP) surrounding the mammalian oocyte acts as a species-specific barrier and is involved in sperm binding. After fertilization, the zona plays a role in blocking polyspermy. It also protects the integrity of the pre-implantational embryo during early embryonic development and contributes to its oviductal transport. In vivo, zona hardening occurs naturally after fertilization in many mammals, included pigs, in order to ensure such three-fold function (De Vos and Van Steirteghem, 2000). This extracellular block to polyspermy is established after biochemical modifications in the ZP due to exocytosis of the cortical granule (CG) contents into the perivitelline space after fertilization (Abeydeera, 2000). Because polyspermic penetration of the vitellus results in embryonic abnormalities in most species, CG release is crucial for normal development (Kola and Trounson, 1989).

Under physiological conditions, porcine oocytes enter the oviduct and are fertilized while enveloped by their cumulus cells (Harper, 1988). Oviductal and cumulus cells exert different actions on the ZP. On the one hand, it has been suggested that fetuin, a component which inhibits ZP hardening during oocyte maturation (Landim-Alvarenga et al., 2002) is produced by granulosa cells to maintain the ZP in a penetrable state for fertilization (Hoyer et al., 2001). On the other hand, the composition of ZP glycoproteins changes during transport through the oviduct (Brown and Cheng, 1986; Hedrick et al., 1987) and oocytes in contact with oviductal secretions need a long time to dissolve the ZP (Broermann et al., 1989). Under in vitro conditions the cortical reaction in porcine oocytes matured and fertilized in vitro has been reported to be delayed (Wang et al., 1997a), and it has been recently suggested that after in vitro fertilization (IVF) hardening of the zona pellucida may not occur (Coy et al., 2002).

In porcine IVF systems, the use of cumulus-enclosed or denuded oocytes is not standardized and is usually determined by the type of spermatozoa employed. Cumulus-enclosed oocytes are usually chosen for refrigerated semen whereas denuded or partially denuded oocytes are used in combination with frozen semen. In this sense, frozen epididymal spermatozoa have some advantages over frozen ejaculated sperm and deliver more consistent in vitro penetration rates with low variability (Rath et al., 1999). Furthermore, scarce information is available about the ZP hardening and cortical reaction of denuded or cumulus-enclosed oocytes and the possible role of oviductal cells during fertilization influencing the further CG exocytosis. However, it is known that the presence of cumulus and oviductal cells at the time of IVF increases the penetrability of oocytes fertilized with epididymal spermatozoa (Romar et al., 2003) and that pre-incubation of oocytes with an oviductal purified glycoprotein (Kouba et al., 2000) and oviductal cells (Kano et al., 1994; Romar et al., 2001) increases monospermy after insemination.

The present study was designed to evaluate the effects of oviductal cell monolayers added before and during IVF using denuded or cumulus-enclosed oocytes to investigate ZP hardening and CG density and distribution.

2. Materials and methods

2.1. Culture media

Unless otherwise indicated, all the chemicals used in this study were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). The medium used for oocyte maturation was NCSU-37 (Petters and Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/ml insulin, 50 μM β-mercaptoethanol, 1 mM glutamine, 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), 10 ng/ml EGF and 10% (v/v) porcine follicular fluid.

The fertilization medium was modified TALP (Rath et al., 1999) supplemented with 3 mg/ml fatty acid-free BSA and 1.10 mM Na-pyruvate.

Oviductal epithelial cells were cultured in TCM 199 with Earle's salts, L-glutamine and $NaHCO_3$ supplemented with 13% (v/v) of fetal calf serum, 150 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Paisley, UK).

2.2. Oocyte collection and in vitro maturation

Ovaries of pre-pubertal gilts were transported at 37 °C from a local abattoir to the laboratory in physiological saline containing 100 mg/ml kanamycin. Cumulus-oocyte complexes were collected from non-atretic follicles (3–6 mm in diameter) by aspiration, and washed twice in modified Dulbecco's phosphate buffered saline (DPBS) supplemented with 1 mg ml^{-1} polyvinyl alcohol (PBS). Oocytes with evenly granulated cytoplasm and several layers of cumulus oophorus cells were selected and rinsed twice in maturation medium, previously equilibrated for at least 3 h under 5% CO_2 in maximally humidified air at 38.5 °C. Groups of 50 oocytes were matured in 500 μl maturation medium for 22 h under 5% CO_2 in air at 38.5 °C. After the maturation period, the oocytes were washed three times and transferred to hormone-free maturation medium for another 22 h (Funahashi and Day, 1993).

2.3. Culture of porcine oviductal epithelial cells (POEC)

The procedure used to culture POEC was as described by Ouhibi et al. (1991) with the following minor modifications. Oviducts from pre-pubertal gilts were recovered from slaughterhouse material. They were rinsed once in saline and twice in PBS at 37 °C in the laboratory. In a Petri dish, fat pads and connective tissues were mechanically removed from the oviducts with sterile forceps and fine scissors. The oviducts were closed at one end with a clip, filled with a trypsin-EDTA solution for endothelial cell culture (500 BAAE units of porcine trypsin and 180 μg EDTA), closed at the other end and incubated for 45 min at 38.5 °C (Romar et al., 2001).

After incubation, the wall of the oviduct was gently squeezed and its contents flushed into a Petri dish containing pre-equilibrated cell culture medium. The epithelial cell clusters were dissociated by gentle, repeated pipetting followed by centrifugation at 800 × *g* for 4 min. The supernatant was discarded and the pellet re-suspended with fresh cell culture medium and seeded at a final concentration of approximately 10^5 cells/well into four-well Nunc plates and cultured at 38.5 °C under 5% CO_2. The medium was changed after 72 h and again every 2 days. Cells reached confluence 5–7 days after initial seeding. Indirect immunocytochemistry tests with a monoclonal antibody raised against cytokeratin (Cox and Leese, 1997; Reischl et al., 1999) were used to ensure the purity of the cell culture following the protocol previously described (Romar et al., 2003).

2.4. *In vitro fertilization*

Epididymal semen was processed for freezing as described by Rath and Niemann (1997). On the day of IVF, three 0.25 ml straws of frozen semen samples were thawed (20 s at 38 °C), diluted in 9.25 ml Androhep® (Minitüb, Tiefenbach, Germany) and centrifuged at 800 × *g* for 3 min. The supernatant was discharged and the sperm pellet re-suspended in modified TALP medium equilibrated overnight in an incubator at 38.5 °C under 5% CO_2. The final sperm concentration was set to 1500 spermatozoa per oocyte in 10 μl. Semen was added to the medium containing the oocytes equilibrated for 30 min before insemination.

2.5. *Assessment of zona pellucida solubility*

At the end of in vitro maturation, oocytes were distributed for the experiment in groups of 10 (IVM group) after pre-incubation for 3 and 6 h with or without POEC (Experiment 1) and 3 h after IVF with or without POEC (Experiment 2). Oocytes were transferred into PBS, washed by pipetting to remove surrounding cumulus cells and attached spermatozoa in the IVF groups, and introduced into 100 μl of 0.1% (w/v) pronase solution in PBS (Kim et al., 1996). Zonae pellucidae were continuously observed for dissolution under an inverted microscope at room temperature. The dissolution time of the zona pellucida of each oocyte was registered as the time interval between placement of the samples in pronase solution and disappearance of the ZP at magnification of 200×.

2.6. *Assessment of cortical granules*

Methods of cortical granule (CG) visualization were based on those described by Yoshida et al. (1993) with modifications reported by Wang et al. (1997b). Briefly, the oocytes were denuded mechanically by repeated pipetting and washed in PBS. Thereafter, oocytes were fixed with 3.7% (w/v) paraformaldehyde in PBS for 30 min at room temperature and washed three times in PBS for 5 min. This was followed by treatment with 0.1% (v/v) Triton X-100 in PBS for 5 min and washing twice in PBS for 5 min. Oocytes were then incubated in 100 μg/ml FITC-PNA in PBS for 30 min in the dark. After staining, the oocytes were washed three times in PBS, mounted on slides with a cover slip, secured by

two lines of vaseline, and sealed with nail polish. Groups of 5 oocytes were analysed at the end of the in vitro maturation period (IVM group), after pre-incubation for 3 and 6 h with or without POEC (Experiment 1), and 3 h after insemination with or without POEC (Experiment 2).

The slides were examined with a Leica DMRB microscopy with a TCS NT confocal module equipped with a Krypto-argon ion laser for excitation of fluorescein to identify CG. Images were recorded digitally on a magnetic optical disk using a Leica PL APO UV 63 × 1.32NA oil objective. In each oocyte two sections were examined under the confocal microscopy. The first one corresponding to the largest diameter section was referred as equator. The second one was taken at 35–40 μm from the equator (Yoshida et al., 1993; Kim et al., 1996) and was referred to as cortex. The images from each of this planes was recorded with software attached to the microscopy being the thickness of each section 1 μm.

Cortical granule density of each oocyte was estimated using image analysis software MIP4 (Consulting of digital imaging S.L., Microm, Spain) following the methodology described by Coy et al. (2002). Firstly it was edited a “macro” consisting in a sequential order for the program to execute the same steps in each oocyte. First, in the equatorial image, two concentric circles were drawn by hand to select the PNA-FITC stained space (area taken by CG) in the space immediately below the oocyte plasma membrane (Fig. 1a and b). The total area selected as well as the marked area were calculated by the software. The area fraction (AF) was calculated as the CG labelled area divided by the entire marked area × 100. In the cortical image, one circle was drawn to isolate the cortex area and the same measure of AF was calculated (Fig. 1c and d). The same macro was run for each oocyte in the different experimental groups.

2.7. Experimental design and statistical analysis

Experiment 1 investigated the effect of oocyte pre-incubation with POEC on ZP hardening and CG density and distribution using cumulus free and cumulus-enclosed oocytes (Fig. 2). After an IVM period (IVM group), a group of oocytes was denuded, one half processed for ZP digestion and the other half for CG study. The remaining matured oocytes were either pre-incubated for 3 and 6 h with oviductal epithelial cell monolayers pre-equilibrated for 1 h with modified TALP medium (POEC group), or were maintained in modified TALP medium in absence of oviductal cells (Control group). At the end of the culture period, oocytes were processed for ZP digestion and CG study. This experiment was carried out over 5 replicates.

In Experiment 2 the effect of POEC was studied during IVF with denuded and cumulus-enclosed oocytes on the ZP hardening and CG density and distribution (Fig. 2). After the maturation period (IVM group), an aliquot of oocytes was denuded and one half processed for ZP digestion, the other half for CG study. All remaining matured oocytes were washed twice in modified TALP medium, transferred to a four-well dish and fertilized in presence (POEC group) or absence (Control group) of a monolayer of oviductal cells equilibrated for 1 h with 190 μl of TALP. Denuded and cumulus-enclosed oocytes were used in both groups. Three hours after insemination oocytes were processed for ZP digestion and CG study. This experiment was carried out over 6 replicates.

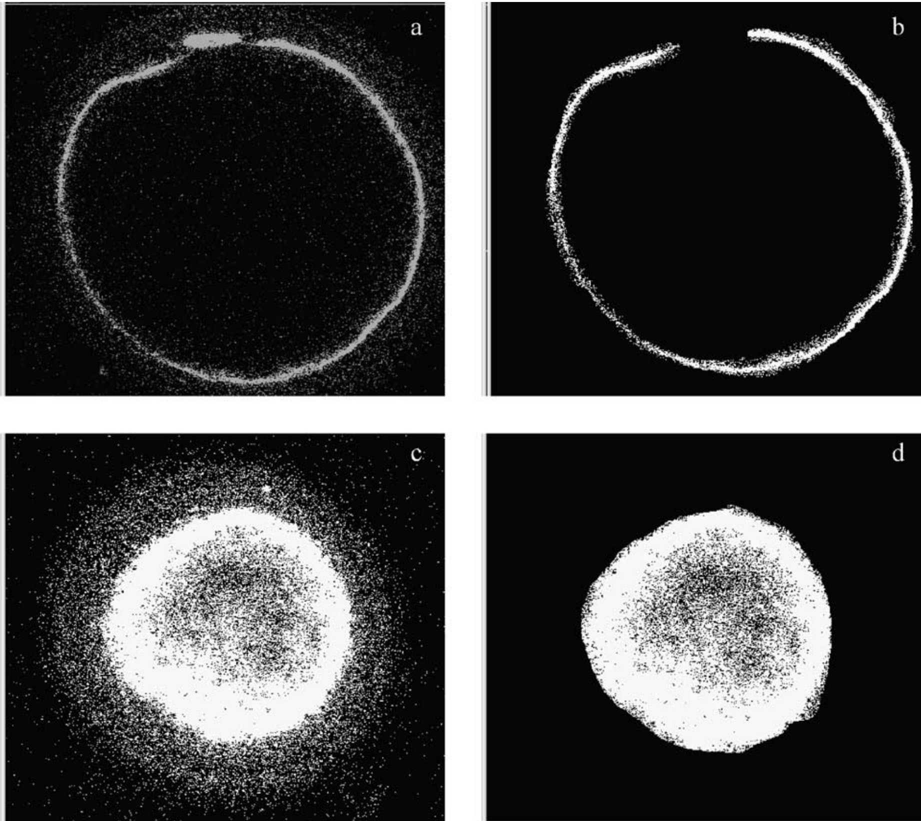

Fig. 1. Defining areas for cortical granule density assessment. (a) Equatorial image of a pig oocyte before isolating the area surrounding the plasma membrane; (b) isolated equator; (c) cortical image of the same oocyte before isolating the cortex area; (d) isolated cortex.

Data are presented as the mean ± SEM, and all rates were modelled according to the binomial model of parameters. In Experiment 1, the variables time of ZP dissolution and percentage of labelled area in the cortex and equator of the oocytes (AF) were analyzed by a three-way ANOVA with the presence of POEC, cumulus cells, time, and their interactions as the main effects. In Experiment 2, variables were analyzed by two-way ANOVA with the presence of POEC, cumulus cells, and their interactions as the main effects. When a significant effect was revealed by ANOVA, values were compared using the Tukey test. A P value < 0.05 was taken to denote statistical significance.

3. Results

Indirect immuno-cytochemistry tests with a monoclonal antibody raised against cytokeratin showed that the purity of epithelial cells in the POEC cultures was at least 95%.

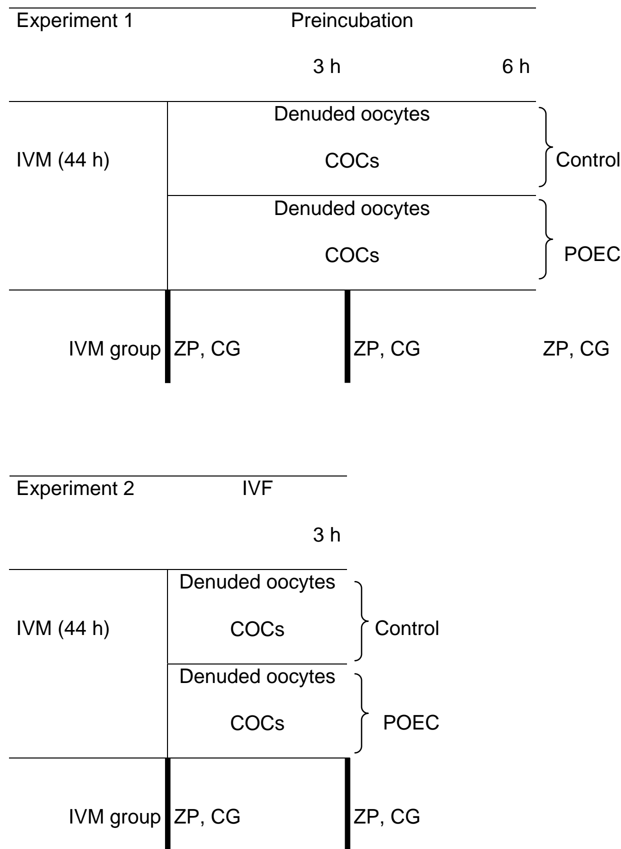

Fig. 2. Schematic depicting experimental design and endpoints. Experiment 1: immediately after IVM oocytes are checked for solubility of the zona pellucida (ZP) and cortical granule (CG; IVM goup). Half of remaining COCs are denuded and both COCs and denuded oocytes are cultured for 3 or 6 h with (POEC) or without (Control) oviductal cells. Experiment 2: Denuded oocytes and COCs are fertilized in presence (POEC) or absence (Control) of oviductal cells for 3 h.

3.1. Effect of oocyte pre-incubation with POEC on ZP hardening and CG density and distribution (Experiment 1)

The ZP of in vitro matured oocytes was digested within 400.46 ± 61.75 s and no differences were observed in the groups ($P = 0.397$, Table 1). Neither the presence of oviductal cells nor of cumulus cells during the pre-incubation time affected ZP digestion with pronase. Moreover, time did not have an influence on ZP hardening and the digestion times were similar among oocytes pre-incubated for 3 and 6 h.

The distribution pattern of CG in each region studied was similar between groups; a higher density was recorded in the cortex area than in the equator. The area fraction (AF) taken by the CG in the equator of in vitro matured oocytes was $22.5 \pm 2.5\%$ and neither POEC nor cumulus cells had an effect on the distribution pattern. However, a significant

Table 1
Effect of pre-incubating denuded and cumulus-enclosed oocytes for 3 and 6 h with or without POEC on the time of zona pellucida digestion

Pre-incubation	Cumulus	Pre-incubation 3 h		Pre-incubation 6 h	
		N	ZP digestion (s)	*N*	ZP digestion (s)
Control	No	46	484.3 ± 50.2	46	464.0 ± 70.4
	Yes	47	561.4 ± 76.3	46	437.1 ± 70.2
POEC	No	46	422.7 ± 33.1	46	411.0 ± 44.2
	Yes	44	568.6 ± 66.4	46	452.1 ± 51.4

N: number of oocytes; IVM: ZP digestion time of oocytes just after IVM. IVM: $N = 50$; ZP digestion = 400.46 ± 61.75 s.

effect of exposure time was observed. AF were bigger in the equator of the 6 h than in the 3 h group ($P < 0.001$), ranging from 15 to 24% in the 3 h group and from 38 to 48% in the 6 h group (Fig. 3, Equator). Oocytes pre-incubated for 6 h had a higher density of CG than oocytes investigated directly after IVM (47.6, 44.7 and 44.7% versus 22.5%; $P < 0.001$), excepting the group of cumulus-enclosed oocytes (37.9%).

AF in the cortex of in vitro matured oocytes was 44.4 ± 3.6%. Both POEC ($P = 0.046$), cumulus cells ($P = 0.005$), and time ($P < 0.001$) had a significant effect on the cortical AF (Fig. 3, Cortex). Besides, the observed interactions between oviductal and cumulus cells were significant ($P = 0.01$). Oocytes cultured in the presence of oviductal cells had a lower AF than those cultured in absence of POEC, cumulus-enclosed oocytes had a lower AF than the denuded ones and oocytes of the 3 h group had a less AF than those of the 6 h group. Only the denuded oocytes cultured for 6 h in absence of POEC were different from the IVM group (70.2 and 44.4% respectively, $P < 0.001$).

3.2. Effect of POEC during IVF on ZP hardening and CG density and distribution (Experiment 2)

Neither the presence of oviductal cells nor of cumulus cells during IVF affected the ZP digestion assessed 3 h after insemination (Table 2, $P = 0.497$).

Cortical granule density in the in vitro fertilized oocytes did not reveal significant differences among groups (Fig. 4). However, AF in equator and cortex was significantly

Table 2
Effect of POEC monolayers during fertilization on the time of zona pellucida digestion 3 h after IVF of denuded and cumulus-enclosed oocytes

IVF	Cumulus	*N*	ZP digestion (s)
Control	No	58	369.8 ± 43.5
	Yes	58	317.6 ± 25.7
POEC	No	58	378.96 ± 26.0
	Yes	57	405.87 ± 38.2

N: number of oocytes; IVM: ZP digestion time of oocytes just after IVM. IVM: $N = 28$; time of ZP digestion = 366.3 ± 55.1 s.

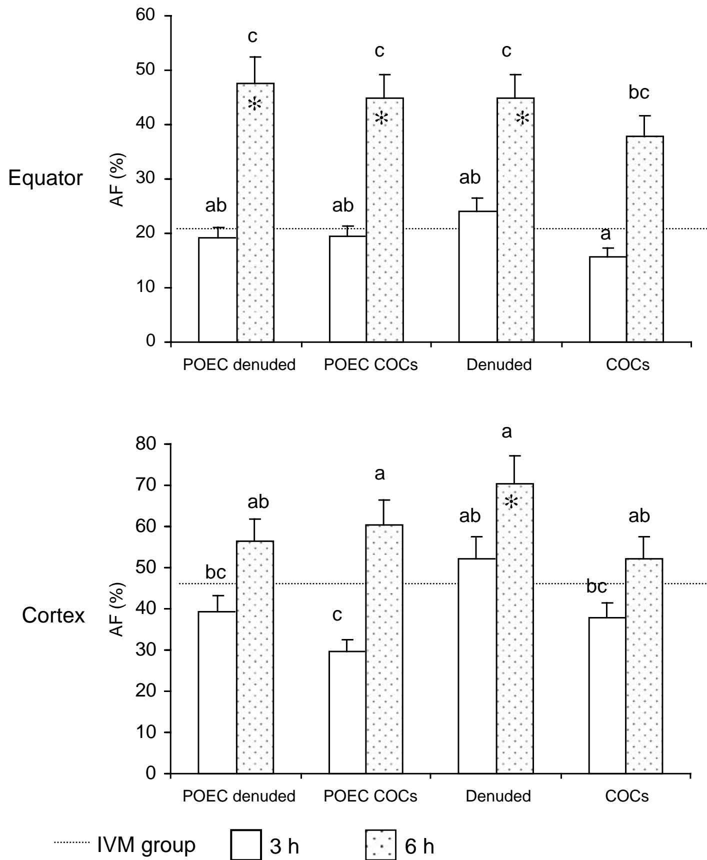

Fig. 3. Changes in cortical granule density (area fraction, AF) in the equator and cortex of cumulus-enclosed (COCs) and denuded oocytes pre-incubated for 3 and 6 h with or without POEC. Different letters (a, b, c) between bars indicate values significantly different ($P < 0.001$), asterisk indicate different from IVM group ($P < 0.001$).

lower in all in vitro fertilized oocytes as compared to oocytes of the IVM group ($P < 0.001$).

4. Discussion

The zona pellucida (ZP) is involved in osmotic regulation, sperm attachment, induction of the acrosome reaction, block to polyspermy, containment of blastomeres and improved survival in the oviduct (Broermann et al., 1989). Under in vivo conditions, oviductal secretions modify its composition and after fertilization undergoes a hardening, which is progressively reversed when the embryo enters the uterus. The necessary time to dissolve the ZP in the in vivo matured oocytes collected directly from the follicles was 6 min whereas, after they enter the oviduct, the digestion time increases upto 21.4 h (Broermann et al., 1989). However,

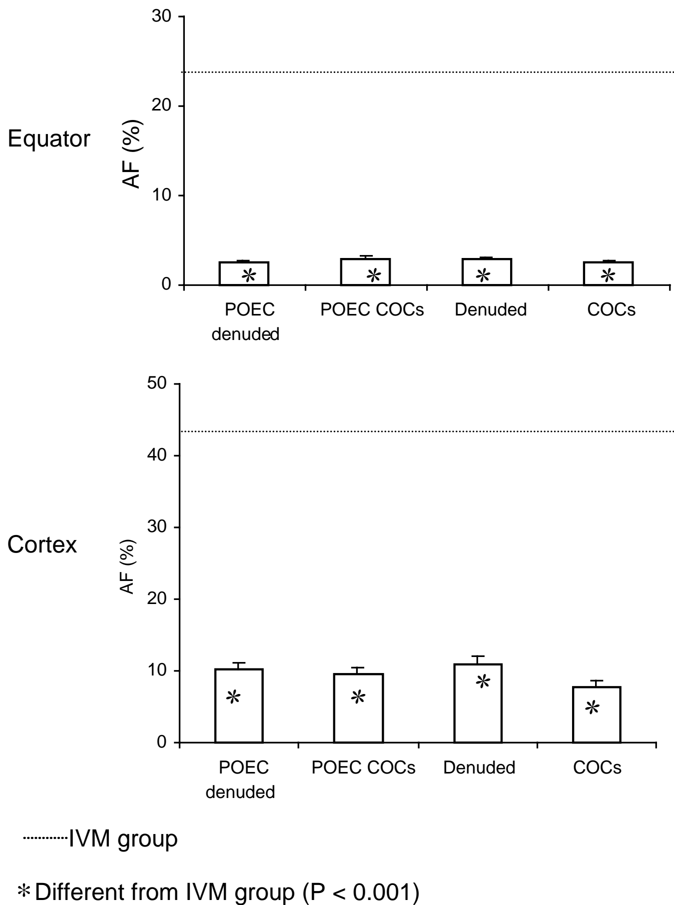

Fig. 4. Changes in cortical granule density (area fraction, AF) in the equator and cortex of cumulus-enclosed (COCs) and denuded oocytes 3 h after fertilization with or without POEC.

when oocytes have been assessed directly after in vitro maturation, different times of ZP dissolution have been reported ranging from slightly than 1 min (Kim et al., 1996) to 7 min (Kouba et al., 2000; Coy et al., 2002). The results of the present study are in agreement with these last data, since the ZP assessed directly after IVM needed a little more than 6 min to be dissolved (Table 1). However, neither contact with POEC nor the presence of cumulus cells during the incubation time changed the time of ZP dissolution. Kim et al. (1996) reported an increase in the time of ZP digestion under in vitro conditions after culturing the oocytes directly in the presence of 30% in vivo collected oviductal fluid. It could be that under our conditions, the responsible factor(s) of such a ZP hardening would have not been secreted to the medium or would have been present at an ineffective concentration during culture. Although oviductal cultures in vitro do not substitute for the oviductal tissue in vivo, an appropriate hormonal stimulation could help to mimic more accurately the physiological

environment. More studies are necessary, especially because Kouba et al. (2000) did not observe a ZP hardening in oocytes matured in vitro and treated with a porcine oviductal purified glycoprotein.

Another possible explanation for the fact that we have not detected an effect on ZP hardening in either kind of studied cells could be that the selected times of culture did not allow us to discriminate an effect, a lack of sensitivity of this test to detect small modifications at the ZP level or the culture medium employed. Regarding the time, an effect was not observed and in vitro matured oocytes reacted similarly to those cultured for additional 3 and 6 h. In mouse (De Felici and Siracusa, 1982) and human (Manna et al., 2001) a "spontaneous hardening" of the ZP was reported in those oocytes maintained in culture. However, the present data seem to indicate that this is different in porcine oocytes.

After fertilization, hardening of the ZP was not observed in any of the groups (Table 2). This lack of hardening after in vitro fertilization of the porcine oocytes has already been described by Coy et al. (2002). This is the opposite to what happens under in vivo conditions (Broermann et al., 1989; Wang et al., 1998) and could be accepted as one of the several factors that affect polyspermic fertilization. This finding is not the only difference between the ZP from in vivo and in vitro porcine oocytes since the last ones have a lower thickness and a higher number of small depressions and "holes" in the zona surface (Funahashi et al., 2000). Summarising, the lack of ZP hardening after IVF could be one explanation for high polyspermic rates in porcine IVM/IVF systems.

In mammals, sperm penetration triggers oocyte activation and subsequent exocytosis of cortical granules (CG). These membrane-bound organelles are synthesized during the maturation period and migrate to the periphery of the matured oocytes at the time of completion of germinal vesicle breakdown and formation of the first metaphase (Cran and Cheng, 1985). In our study, the area fraction (AF) occupied by CG between the oocytes after 44 h of in vitro maturation and the 3 h pre-incubated ones was similar (Fig. 3). However, a higher density of CG was observed when oocytes remained in culture for 6 h. Coy et al. (2002) have also shown an increase in the density of CG when porcine oocytes are kept in culture for 5 h after IVM. This increase in the CG density during the time could be explained by a continuous formation of CG as it has been demonstrated in some species such as the rabbit (Guraya, 1985). In vivo, cortical granules increase in number during the late stages of oocyte maturation. In porcine, up to 20 h following hCG, less than 20% of cortical granules may be detected beneath the plasma membrane but this figure increases to 75% from 30 to 40 h and by 50 h (8–10 h post-ovulation) virtually 100% have migrated to form a monolayer (Cran and Cheng, 1985). In our study the 6 h pre-incubation period after IVM (44 h) would correspond to 50 h from the "artificial" LH surge. Thus, the observed increase in CG density is consistent with the in vivo situation. Although this higher availability of CG would presumably lead to a decrease in the polyspermy, the fertilization of these oocytes showed high penetrability and polyspermy results (Romar et al., 2003). Regarding the effect of cumulus cells on CG density, cumulus-enclosed oocytes had a lower area fraction (Fig. 3) that could explain the higher penetrability of COCs compared to denuded oocytes (Romar et al., 2003). However, the beneficial direct action of cumulus and oviductal cells on the penetrability of spermatozoa could also explain these results.

It has been suggested that the high polyspermy observed in porcine oocytes matured in vitro is due to a delay in CG exocytosis (Wang et al., 1997a). However, examination

of in vitro and ovulated oocytes by confocal microscopy at 6 h after fertilization revealed no difference in CG release (Wang et al., 1988). In our study, after in vitro fertilization all experimental groups showed AF values in the equator and cortex at a lower degree as compared to non-fertilized oocytes (Fig. 4). This important decrease in the CG density may correspond to the cortical reaction. In hamster, CG exocytosis takes 9 min to be completed (Stewart-Savage and Bavister, 1991) and in mouse, fluorescence microscopy indicates that exocytosis starts within 5–10 min and continues upto an hour (Tahara et al., 1996). In pigs, it has been observed that 58% of the oocytes showed no signs of CG exocytosis 6 h after insemination with ejaculated boar spermatozoa semen (Wang et al., 1997a) and Coy et al. (2002) showed that IVF medium affects the CG density. We observed that majority of oocytes had an important decrease in CG density 3 h after insemination with epidydimal semen, both in equator and cortex levels. Again, no effect of POEC or cumulus cells was observed, suggesting that under the employed conditions, denuded and cumulus-enclosed oocytes show similar cortical reaction.

In conclusion, oviductal and cumulus cells did not affect ZP hardening under the employed conditions nor CG density, although an increase in CG density was observed when oocytes were maintained in culture. After fertilization, no ZP hardening was observed and denuded and cumulus-enclosed oocytes showed a similar cortical reaction after IVF with epididymal spermatozoa regardless of presence of POEC.

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