The effect of oviductal epithelial cell co-culture during in vitro maturation on sow oocyte morphology, fertilization and embryo development

A Kidson¹, EJ Schoevers¹, P Langendijk², JHM Verheijden¹, B Colenbrander¹, MM Bevers¹

¹Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584CL Utrecht, The Netherlands ²Department of Animal Sciences, Wageningen Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Abstract

In vitro embryo production in the sow is challenged by poor cytoplasmic maturation, low sperm penetration and low normal fertilization, leading to the development of poor quality blastocysts containing a small number of nuclei. In prepubertal gilt oocytes, the presence of porcine oviductal epithelial cells (pOEC) during maturation increases cytoplasmic maturation and blastocyst development. These aspects, as well as blastocyst quality, may be improved when adult sow oocytes are matured with pOEC. Therefore, the effect of the presence of pOEC on sow oocyte morphology, fertilization and the progression of embryo development was evaluated. The pOEC were cultured in M199 for 18 h, then cultured in NCSU23 for 4 h before the oocytes were added. Oocytes from 2 to 6 mm follicles were matured in 500 ml NCSU23, with eCG and hCG, for 24 h, and then cultured with or without pOEC, in NCSU23 without hormones, for 18 h. In vitro fertilization took place in modified Tris-buffered medium, for 6 h, and the presumptive zygotes were then cultured for 162 h in NCSU23. Morphology of the IVM oocytes was compared to that of immature oocytes and in vivo matured MII oocytes from slaughtered sows in estrus. The in vitro matured oocytes had a greater diameter and a wider perivitelline space than the immature and in vivo matured MII oocytes (P<0.01). Penetration, polyspermy and pronucleus formation did not differ between the pOEC and Control groups, although the total penetration rate was higher for the Control oocytes (26% versus 39%; P<0.01). Fewer blastocysts developed in the pOEC group than in the Control group (19% versus 27%; P<0.01), but blastocyst growth was accelerated, leading to a higher percentage of hatched blastocysts (3% versus 10%; P<0.01). Finally, the average blastocyst cell number was higher in the pOEC group (47 versus 40; P<0.05) and a greater percentage of blastocysts contained a superior number of nuclei. In conclusion, the addition of pOEC during the second half of in vitro maturation resulted in fewer blastocysts formed, but of those blastocysts that did form, the quality was improved.

Theriogenology 2003 59: 1889-1903
Introduction

In vitro embryo production (IVP) is an important tool for study of the regulation of oocyte maturation and early embryonic development in the pig. During the past decade effective porcine IVP systems have been developed, leading to blastocyst development rates comparable to that of other domestic species such as the bovine and ovine [1-3]. Unlike other domestic species though, pig IVP is challenged by serious problems such as incomplete cytoplasmic maturation [1,4], leading to the development of blastocysts with reduced cell numbers and compromised viability [5]. Two recent studies have also demonstrated distinct differences in the developmental competence of prepubertal gilt and adult sow oocytes [4,6]. Although polyspermy has generally been regarded as the major obstacle in porcine IVF, these studies revealed greatly reduced incidences of polyspermy in oocytes from adult sows, but this was accompanied by lower rates of penetration and normal fertilization. Nevertheless, a higher proportion of oocytes developed to blastocysts from sow oocytes than from prepubertal gilt oocytes [4]. These studies indicate different requirements for the in vitro maturation and in vitro fertilization environments for prepubertal gilt and sow oocytes.

After ovulation, the oocyte resides in the oviduct a few hours before fertilization occurs. In vitro matured prepubertal gilt oocytes display different morphological and fertilization characteristics from those of ovulated oocytes [7]. A preliminary study by Day et al. [8] indicated that in vitro matured prepubertal gilt oocytes exposed to the estrous oviduct had a more similar morphology to ovulated oocytes, associated with lower polyspermic penetration. Co-culturing the oocytes with homologous oviductal epithelial cells [2,9,10] or adding oviductal fluid to the culture medium [11] increased normal fertilization and reduce the incidence of polyspermy in oocytes from prepubertal gilts. The effect of porcine oviductal epithelial cells (pOEC) during maturation on subsequent embryo development was examined by Bureau et al. [2] who found a pOEC-induced increase in the rate of blastocyst development compared with the pOEC-free control group. Whether pOEC co-culture also improves blastocyst quality by increasing the number of nuclei has not yet been established.

In vitro produced porcine embryos are retarded in their developmental progression compared to their in vivo counterparts as judged by the number of nuclei per blastocyst [5,11-14]. Improved cytoplasmic maturation of prepubertal gilt oocytes [1,13] results in improved blastocyst development rates, but the relation to blastocyst quality or number of nuclei is not fully established. Hatching of in vitro produced blastocysts is also rarely achieved, and requires the presence of serum and amino acid supplementation of the culture medium [15-18]. In the mouse, IVM/IVF derived blastocysts with reduced cell numbers are less viable after transfer and rarely lead to pregnancies [19-22]. This brings to light the necessity for acceleration of growth or cell division and increase of cell numbers of in vitro blastocysts in order to achieve blastocysts of quality equal to those produced in vivo.

The addition of pOEC during in vitro maturation is known to increase normal fertilization [2,8-10], but their effect on cytoplasmic maturation of sow oocytes, related to blastocyst development and specifically blastocyst quality, has not been determined. In this study we hypothesized that pOEC co-culture during IVM would improve cytoplasmic maturation of the oocyte, leading to increased fertilization rates, blastocyst quality and development. We therefore investigated the effects of pOEC co-culture during in vitro
maturation on oocyte morphology, fertilization parameters, the subsequent progression of embryo development and resultant blastocyst quality.

**Materials and methods**

**Culture media**

For oviduct trimming, oocyte searching and selection, 25 mM Hepes-buffered Tyrode's medium containing 0.1% polyvinyl alcohol (TL-Hepes-PVA) was used. The pOEC were harvested and washed in Hepes-buffered M199 (Gibco BRL, Paisley, UK). Culture of the pOEC took place in M199 supplemented with 2.2 mg/ml NaHCO3 with 75 mg/ml potassium penicillin G and 50 mg/ml streptomycin sulfate (PenStrep; Sigma, St. Louis, MO, USA). Cumulus-oocyte-complexes (COCs) were washed in IVM-wash medium consisting of NCSU23 medium [18] supplemented with 10% porcine follicular fluid (pFF), 0.57 mM cysteine, 25 mM β-mercaptoethanol and 20 mM Hepes. The maturation medium for the first 24 h of in vitro maturation, designated IVM-I, was NCSU23 with 10% pFF, 0.57 mM cysteine, 25 mM β-mercaptoethanol, 10 IU/ml eCG (Chorulon, Intervet, Boxmeer, The Netherlands) and 10 IU/ml hCG (Folligonan, Intervet, Boxmeer, The Netherlands). For the second 18-20 h of maturation (IVM-II), the same medium was used as for IVM-I, but without eCG and hCG and supplemented with PenStrep. The fertilization medium (IVF-medium) was modified Tris-buffered medium (113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM D-glucose, 7.5 mM CaCl2·2H2O and 5.0 mM Na-Pyruvate) containing 1 mM caffeine and 0.1% BSA (Sigma, St. Louis, MO, USA). In vitro culture of the zygotes then took place in NCSU23 medium supplemented with 0.4% BSA (IVC-medium).

**Preparation of porcine oviductal epithelial cells**

Oviducts attached to ovaries with recent corpora were used for obtaining epithelial cells. The age of the corpora hemorrhagica was estimated following previous experience using oviducts from sows slaughtered soon after ovulation. Such oviducts display vigorous ciliary and secretory activity, known to be indicative of viability [23,24]. The method described below was developed during a prestudy in this laboratory. Two oviducts were collected from two different sows at the slaughterhouse and transported to the laboratory at 27-30 °C within 3 h postmortem. The oviducts were then dissected free of the ovaries and other tissues, and washed twice in 0.9% saline with 75 mg/l potassium penicillin G and 50 mg/l streptomycin sulfate at 30 °C before sectioning. Approximately 3 cm from the infundibulum (ampullar region) two sections of 1 cm each were cut off each oviduct and washed once more in room temperature saline. The sections were carefully wiped to remove excess saline and placed in room temperature M199-Hepes with 10% FCS and PenStrep. The pOEC were obtained by slicing the oviduct longitudinally and scraping the inside using a scalpel blade, keeping the oviduct immersed in the medium. The pOEC were then flushed in and out of a 21 g needle three times in order to separate the cells into smaller clumps. The cells were then centrifuged at 300 x g for 3 min, the supernatant removed, the pellet resuspended and the procedure repeated twice. After the final centrifugation, the soft pellet was resuspended in 1 ml M199-Hepes with 10% FCS and PenStrep and 20 ml of this cell suspension was transferred to 500 µl M199 with 10% FCS and antibiotics in a 4-well culture dish. The dish was then incubated at 38.5 °C in 5% CO2 for 18 h. After the incubation period, the contents of each well were removed and
placed in a 15 ml polystyrene conical centrifuge tube. Each of the wells, which contained some attached oviductal cells, were then washed three times using 500 µl IVM-wash medium. After the final wash, 500 µl IVM-II medium was added and covered with warmed mineral oil. The pOEC were then washed three times by centrifugation at 300 x g using prewarmed IVM-wash medium. After the final wash, the pellet was resuspended in 80 ml IVM-II medium and 20 ml of this suspension was added to each well. The pOEC culture dish was then incubated for 4 h before the COCs were added for IVM-II.

Oocyte maturation in vitro

Ovaries, and attached oviducts, were collected from sows after slaughter and transported to the laboratory in an insulated container within 3 h postmortem at 27-30 °C, the temperature of the ovaries upon collection at the slaughterhouse. Upon arrival, the ovaries were washed under running tap water at 27-30 °C, then dried gently using paper towels and placed in 0.9% saline with 75 mg/l potassium penicillin G and 50 mg/l streptomycin sulfate at 27-30 °C until aspiration. COCs were aspirated from 2 to 6 mm follicles using an 18 g winged infusion set needle attached to a 50 ml polystyrene conical tube by means of a suction pump under pressure. Follicular contents were allowed to settle at room temperature after which the supernatant was discarded and the sediment resuspended with TL-Hepes-PVA. This procedure was repeated twice. Oocytes surrounded by three or more layers of compact cumulus cells were selected irrespective of ooplasm appearance and washed twice in IVM-wash medium. Then 45-50 oocytes were cultured in each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 500 µl of IVM-I medium which had previously been covered with mineral oil and equilibrated for 2 h at 38.5 °C and 5% CO₂ prior to use. After 24 h of maturation, the COCs were washed twice in IVM-wash medium and placed in IVM-II medium, after which they were cultured for an additional 18-20 h.

Fertilization and embryo culture in vitro

Fresh BTS-extended (Beltsville Thawing Solution) [25] semen from two randomly selected boars, irrespective of breed, was obtained from the local AI station. The spermatozoa were allowed to cool to room temperature for 30 min before processing. 500 ml sperm from each boar was pooled together in a 15 ml polystyrene conical tube (Greiner) and 2 ml room temperature IVF-medium (previously equilibrated for 18 h at 38.5°C in 5% CO₂) was added. After centrifuging at 700 x g for 5 min at room temperature, the sperm pellet was resuspended to 1 ml IVF-medium. This procedure was then repeated, the concentration adjusted to 8 x 10⁴ motile sperm/ml, and the sperm suspension warmed to 38.5 °C for 15 min before being added to the IVF drops.

After maturation was completed, the expanded cumulus cells were removed by pipetting. The denuded oocytes were washed twice in IVF medium, and 45-50 oocytes were placed in 50 ml drops of IVF medium that had previously been covered with mineral oil and equilibrated at 38.5 °C in 5% CO₂ in a 35 mm x 10 mm petri dish (Nunc, Roskilde, Denmark) for 1 h. For fertilization, 50 ml of the diluted sperm suspension were added to each 50 µl drop containing the oocytes, giving a final sperm concentration of 400,000 sperm/ml. The spermatozoa and oocytes were co-incubated for 6 h at 38.5°C in 5% CO₂ humidified air. The oocytes were then washed in IVC-wash medium and transferred to IVC-medium for 12 h to 7 days.
Recovery of in vivo matured oocytes

Oocytes were recovered from the ovaries of GnRH-synchronized sows approximately $3 \pm 8$ h before estimated ovulation. Briefly, at 83-87 h after weaning, sows were treated with 50 mg of GnRH i.m. (Depherelin, Veyx-Pharma, Germany), to synchronize ovulation. Previous preliminary research [26] has shown that in sows treated around this time, ovulation occurs 35-41 h after GnRH administration. These sows were then slaughtered and the ovaries removed at approximately 38 h after GnRH treatment. Oocytes were aspirated from the follicles using an 18 g needle attached to a 10 ml syringe. The follicular contents of each individual syringe were placed in a 90 mm petri dish and approximately 10-12 ml of TL-Hepes-PVA was added to dilute the opaque follicular fluid in order to visualize the COCs clearly. The expanded COCs were then washed twice using TL-Hepes-PVA and placed in a solution of 0.2% hyaluronidase for 5 min to remove the majority of the cumulus cells. The remaining cumulus cells and corona radiata were removed by repeatedly passing the oocyte through a narrow bore glass pipette. The denuded oocytes were washed twice in TL-Hepes-PVA, and an image of each oocyte was recorded as described in Experiment 1. The oocytes were then fixed in 2% paraformaldehyde and stained with 4,6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA; see Experiment 2) to determine the stage of nuclear maturation. Of this group, only oocytes at metaphase II were taken into consideration for morphological evaluations.

Experimental design

Experiment 1: morphological comparison of in vitro and in vivo matured oocytes

In the first experiment, a comparison of morphological characteristics of oocytes from four different origins were made: immature oocytes from 2 to 6 mm follicles (n=23); oocytes after 42-44 h in vitro maturation without pOEC (n=48); oocytes after 24 h in vitro maturation without pOEC and then 18 h with pOEC (n=35); and metaphase II oocytes from pre-ovulatory follicles (three sows; n=49). Immature oocytes were treated similarly to in vivo oocytes for the removal of the cumulus cells. All oocytes were placed in the same medium (TL-Hepes-PVA) for imaging to avoid any differences that might occur due to changes in osmolarity. Evaluations were made of the appearance of the ooplasm, the diameter of the oocyte, the thickness of the zona pellucida (ZP), the size of the perivitelline space and the zona-free diameter of the ooplasm. Images were recorded with a digital camera (Nikon Coolpix 990) at 200 x magnification on an inverted microscope (Olympus IM, Tokyo, Japan), and measurements were made from the images after printing (Fig. 1).

Experiment 2: the effect of pOEC co-culture during IVM on sperm penetration after in vitro fertilization

Oocytes collected from slaughterhouse ovaries, matured in the absence (42 h without pOEC) or presence of pOEC (24 h without and then 18 h with pOEC), were fertilized in vitro. The sperm penetration parameters were determined 18-20 h after the onset of IVF. Oocytes were washed in TL-Hepes-PVA and fixed in 2% paraformaldehyde for at least 4 days at 4°C, then washed three times in TL-Hepes-PVA before being stained with 0.1 mg/ml DAPI, mounted and observed under epifluorescence at 400 x magnification (BH2-RFCA Olympus, Tokyo, Japan).
Penetration parameters were classified as follows: total penetration rate (TPN), oocytes containing an intact sperm head, a decondensed sperm head, one or more male pronuclei, syngamy, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; penetration rate (PEN), as for TPN, but excluding intact sperm heads; male pronucleus formation (MPN), oocytes containing one or more male pronuclei or two blastomeres each containing a normal nucleus in the presence of two polar bodies; polyspermy, oocytes containing more than one decondensed sperm head or male pronucleus; normal fertilization, monospermic oocytes with two pronuclei and two polar bodies, or a decondensed sperm head, a female pronucleus and two polar bodies, or syngamy with two polar bodies, or two blastomeres each containing a normal nucleus in the presence of two polar bodies. The cytoplasmic index (CI) is defined as the ratio of the percentage PEN to the percentage TPEN. The index has a value between 0 and 1, with higher values implying greater cytoplasmic proficiency of the oocytes.

Experiment 3: in vitro embryo development of in vitro fertilized oocytes following IVM in the presence or absence of pOEC

Oocytes were matured and fertilized as in Experiment 2, but presumptive zygotes were subsequently cultured up to 168 h after insemination. The cleavage rates were assessed at 48 h post-insemination. Only embryos consisting of two to eight evenly sized blastomeres were included. Blastocyst formation and hatching was determined on Day 5, Day 6 and Day 7 after insemination (Day 0). Blastocysts produced were then fixed and stained to determine the number of nuclei. Briefly, blastocysts were washed in TL-Hepes-PVA and fixed in 2% paraformaldehyde for at least 4 days at 4 °C. They were then washed three times in TL-Hepes-PVA before being stained with DAPI and mounted. The number of nuclei was determined by viewing the blastocysts under epifluorescence. An image of each blastocyst was recorded using a digital camera, and the cells were then counted after printing of the images (Figure 2).

Statistical design

At least three replicate trials were carried out for each experiment. Morphological differences were analyzed by ANOVA, followed by the Bonferroni post hoc test. Differences in the rates of total penetration (TPN), penetration (PEN), male pronucleus formation (MPN), polyspermy, cleavage (CR) and blastocyst formation were analyzed by Fisher’s exact test. Blastocyst cell numbers were analyzed by t-test and are presented as mean ± SEM. A difference with P<0.05 was considered to be statistically significant.

Results

Experiment 1

Differences in the distribution of the ooplasm between immature, in vitro matured and in vivo matured oocytes were observed (Fig. 1). The majority (98%) of in vivo matured oocytes displayed a continuous clear space in the cortex of the ooplasm, approximately 12-15 mm deep. Clear spaces 7-10 mm in depth were also seen in the ooplasm of the in vitro matured oocytes, but these were distributed semicontinuously in only 40-60% of the ooplasm cortex. Immature oocytes had an evenly distributed ooplasm with no clear spaces.
Experiment 2

In vitro maturation of the oocytes from adult slaughterhouse sows, irrespective of the treatment, resulted in more than 97% MII oocytes by 42-44 h of IVM (data not shown). The effect of pOEC co-culture on in vitro fertilization is presented in Table 2. A distinction was made between the total penetration rate (TPEN) and the penetration rate (PEN). The TPEN results displayed in Table 2 indicate that significantly more oocytes had been penetrated by spermatozoa in the Control, than in the pOEC group. Of the sperm in the vitellus of the pOEC oocytes a greater proportion had undergone decondensation (CI: 0.44 versus 0.67; for Control and pOEC, respectively) during this time period (18 h) signifying a greater degree of cytoplasmic support of fertilization in this group. No differences were found in the rate of polyspermy nor the percentage of male pronucleus formation.
Table 1. Morphological characteristics of sow oocytes before (immature oocytes) and after IVM in the absence (IVM-II-control) or presence of porcine oviductal epithelial cells (IVM-II-pOEC), compared to in vivo matured MII oocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes measured (n)</th>
<th>Oocyte diameter (µm ± SEM)</th>
<th>Oocyte diameter without ZP (µm ± SEM)</th>
<th>Thickness of ZP (µm ± SEM)</th>
<th>Size of perivitelline space (µm ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>23</td>
<td>146.85 ± 0.92^a</td>
<td>113.14 ± 1.2</td>
<td>14.06 ± 0.28</td>
<td>2.80 ± 0.22^c</td>
</tr>
<tr>
<td>IVM-II-control^1</td>
<td>48</td>
<td>149.45 ± 0.60^b</td>
<td>112.66 ± 0.65</td>
<td>14.15 ± 0.20</td>
<td>4.25 ± 0.21^d</td>
</tr>
<tr>
<td>IVM-II-pOEC</td>
<td>35</td>
<td>150.43 ± 0.64^b</td>
<td>114.10 ± 0.71</td>
<td>14.31 ± 0.37</td>
<td>4.40 ± 0.30^d</td>
</tr>
<tr>
<td>In vivo MII</td>
<td>49</td>
<td>146.96 ± 0.50^a</td>
<td>112.14 ± 0.61</td>
<td>14.82 ± 0.19</td>
<td>2.60 ± 0.12^c</td>
</tr>
</tbody>
</table>

Different superscripts (a, b) within the same column, P<0.01; different superscripts (c, d) within the same column, P<0.001. ^1IVM-II-control: no pOEC co-culture during IVM.

Experiment 3

Porcine OEC co-culture did not have any significant effect on the progression of early embryo development up to Day 5 blastocyst development (Table 3). The percentage of zygotes that had cleaved by Day 2 of in vitro culture (34 and 29% for the Control and pOEC groups, respectively) appeared to be equivalent to the TPEN rate (Table 2; 36% for the Control, and 29% for the pOEC). In the pOEC group, the percentage normal fertilization was also equal to the percentage of zygotes that had progressed to the 4-cell stage by Day 2 after IVF (Table 3). These embryos could be considered to be synchronous and thus at a similar stage of development with their in vivo counterparts [27,28].

As depicted in Table 1, the diameter of the in vitro matured oocytes was significantly greater (P<0.01) than that of immature oocytes from 2 to 6 mm follicles and the in vivo matured MII oocytes. Neither the oocyte diameter without ZP, nor the thickness of the ZP differed between any of the groups. In vitro maturation, with or without pOEC, resulted in a significant increase in the size of the perivitelline space (P<0.01 for in vivo MII versus IVM-II-control and IVM-II-pOEC; P<0.001 for pre-IVM versus IVM-II-control and IVM-II-pOEC). Co-culture of the oocytes with pOEC did not result in any significantly different morphological observations.

Table 2. The effect of pOEC co-culture, during IVM, on fertilization parameters 18 h after IVF of sow oocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes (n)</th>
<th>Total penetration % (TPEN)</th>
<th>Penetration % (PEN)</th>
<th>Cytoplasmic Index**</th>
<th>Polyspermy % (of TPEN)</th>
<th>MPN % (of PEN)</th>
<th>Normal fertilization % (of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVM-II-control^1</td>
<td>193</td>
<td>36^a</td>
<td>17</td>
<td>0.44</td>
<td>4</td>
<td>82</td>
<td>15</td>
</tr>
<tr>
<td>IVM-II-pOEC</td>
<td>174</td>
<td>29^b</td>
<td>21</td>
<td>0.68</td>
<td>8</td>
<td>81</td>
<td>18</td>
</tr>
</tbody>
</table>

Different superscripts (a, b) within the same column, P<0.01. ^1IVM-II-control: no pOEC co-culture during IVM. Cytoplasmic index is defined as the ratio of the percentage PEN to the percentage TPEN.
Furthermore, similarities could be found between the percentage normal fertilization (15% versus 18% for Control and pOEC groups, respectively) and the blastocyst development on Day 5 (15% for both of the groups). These blastocysts appeared to be developing at the same rate as their in vivo complement [27,28]. On Day 6, significantly more embryos were at the blastocyst/expanded blastocyst stage in the Control group than in the pOEC group (24% versus 19%; P<0.01). Similarly, on Day 7 more Control embryos had developed to blastulation compared with those in the pOEC group (27% versus 19%; P<0.01).

In addition, of the pOEC co-cultured blastocysts, 10% had hatched or were hatching, compared to 3% in the Control group (P<0.05), indicating that the rate of embryo growth was also accelerated by the pOEC co-culture. In accordance with the higher hatching rate for pOEC co-cultured blastocysts, these blastocysts on average contained significantly more nuclei than the Control blastocysts (47 ± 2 versus 40 ± 2; P<0.05). Figure 3 demonstrates the distribution of blastocysts according to the number of nuclei contained: blastocyst cell numbers in the Control group were spread symmetrically over the median providing equal-sized intervals to contain the middle quartiles of the cell number data, whereas the distribution of the pOEC co-cultured blastocyst cell numbers were skewed to the right indicating the larger percentage (of the total number of blastocyst in this group) of pOEC co-cultured blastocysts with greater cell numbers. These data therefore indicate that the ratio of blastocysts containing a superior number of nuclei (of the total number of blastocysts in the group), was greater in the pOEC group than in the Control group.

Discussion

In the first experiment of this study we compared morphological characteristics of sow oocytes matured in vitro, in the presence or absence of pOEC, and in vivo. Metaphase II oocytes collected from preovulatory follicles, after an artificially induced LH peak, were compared to MII in vitro matured oocytes and considered to represent their `in vivo complement.' Clear spaces were observed in the cortex of the ooplasm in both the in vivo and in vitro matured oocytes, but not in the immature oocytes which displayed a dense evenly granulated ooplasm. Pubertal gilt oocytes recovered from the oviduct after ovulation have clear areas in the cortex cytoplasm, in contrast with a dense evenly granulated ooplasm evident in in vitro matured oocytes from prepubertal gilts [7]. These differences in the distribution of the ooplasm content may be due to modifications in the cytoskeleton of the oocyte during the final maturation phases in vivo, resulting in an altered organization of organelles within the ooplasm. Nagashima et al. [29] found that oocytes with unevenly granulated ooplasm, after IVM, had greater developmental competence. As the appearance of the pOEC-matured ooplasm did not differ from that of the IVM-Control, and was largely similar to the in vivo matured oocytes, it seems probable that the changes in the distribution of organelles take place in the pre-ovulatory follicle during maturation, or during in vitro maturation. These changes in the aforementioned oocyte characteristics may therefore not be due to the effect of oviductal contact as previously suggested [7], but may indeed be owed to incomplete differentiation of prepubertal oocytes as suggested by Marchal et al. [4].
Figure 2. Digital micrographs (200 x magnification) of in vitro produced Day 7 blastocysts derived from in vitro matured sow oocytes fertilized and cultured in vitro. (A) An expanded Control blastocyst. (B) An expanded Control blastocyst stained with DAPI for cell number determination. (C) A hatching pOEC-blastocyst. (D) A hatching pOEC blastocyst stained with DAPI.

Table 3 The effect of pOEC co-culture, during the second half of IVM, on porcine embryo development from Day 2 to Day 7 following IVF

<table>
<thead>
<tr>
<th></th>
<th>Cleavage Rate on Day 2 (%)</th>
<th>Blastocyst Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>2-cell 4-cell 8-cell Total</td>
<td>Day 5  Day 6  Day 7 Day 7 Hatching</td>
</tr>
<tr>
<td>Control*</td>
<td>358   6    22 6  34</td>
<td>15  24a  27a 3a</td>
</tr>
<tr>
<td>POEC</td>
<td>391   5    18 5  29</td>
<td>15  19b  19b 10b</td>
</tr>
</tbody>
</table>

Different superscripts (a, b) within the same column, P < 0.01. *Control: no pOEC co-culture during IVM.

The perivitelline space of the in vitro matured oocytes was wider than that of the in vivo matured oocytes, and probably accounts for the larger diameter of the in vitro matured oocytes. An increase in the size of the perivitelline space is usually associated with oocyte activation [7]. In mouse, hamster and human oocytes it has been shown [30,31] that a ‘partial cortical reaction’ takes place in oviductal oocytes prior to fertilization; this might play an important role in the conditioning of the ZP prior to the ZP reaction. These oocytes also have a fully developed perivitelline space. Although prefertilization cortical granule release has not yet been identified in pig oocytes, oviductal oocytes have previously been shown to have a wider perivitelline space than in vitro matured...
prepubertal gilt oocytes [7].

Although higher rates of blastocyst formation are found when using sow oocytes, sperm penetration rates are lower compared with oocytes from prepubertal gilts [4,6]. This serves as indication of possible differences in the requirements for both oocyte maturation and IVF conditions for sow as opposed to prepubertal gilt oocytes. In this study we did not find any increase in penetration rates for the pOEC oocytes and the explanation might be found in the absence of the cumulus cells during IVF. Extended physical contact between the oviductal cells and the COC [8] are necessary for achieving higher penetration rates, and it is probable that the effect of the pOEC is lost due to the commonly used procedure of oocyte-denudation before IVF. In vivo, after ovulation, the cumulus surrounding the oocyte is largely intact and groups of ovulated oocytes clump together in a mass of mucified expanded cumulus [32]. It is in this state that spermatozoa encounter the COCs. In the cow and pig, denudation of the oocyte prior to IVF often leads to reduced penetration rates [33,34]. While this practice may be of benefit in prepubertal gilt IVF, where polyspermy is common, it might very well have the opposite effect in sow IVF.

Oviductal proteins are known to be associated with the zona pellucida, perivitelline space and vitelline and blastomere membranes of ovulated oocytes and oviductal embryos [35-37], suggesting a role during fertilization and early embryonic development. The exact function and effect of these proteins, and other oviductal compounds, on oocyte morphology and function have not yet been fully elucidated, but the results presented in Experiment 2 demonstrate an effect of pOEC on the progression of fertilization. In this study, both the total penetration rate (TPEN) and penetration rate (PEN) were taken into account to obtain a clearer picture of the oocyte penetration and the ability of the ooplasm to support sperm decondensation at the designated time point of 18 h after the start of IVF. The cytoplasmic index was hypothesized to provide information on the 'normal' progression of fertilization and served as an indicator of the cytoplasmic proficiency of the oocyte and hence the quality of the resultant zygotes. The CI for the Control group was lower than that of the pOEC group, indicating delay in the decondensation of the sperm found in the vitellus and thus suboptimal functioning of the ooplasm. Factors found in oviductal fluid are known to modulate protein synthesis in zygotes and embryos [36], and pOEC-produced secretions during in vitro maturation may therefore have contributed to the enhanced functioning of the cytoplasm of pOEC-matured oocytes. The parameter generally used to signify the cytoplasmic maturity of the oocyte (percentage pronucleus formation) was not different for the two groups, and provided no correlation with oocyte or embryo quality.

The fact that blastocyst development seemed to have plateaued in the pOEC-treated group and was lower than that of the Control group was in contrast to our hypothesis that pOEC treatment would increase blastocyst development, and also contrary to the results obtained by Bureau et al. [2] who found that pOEC co-culture of oocytes enhanced the subsequent percentage of oocytes developing to blastocysts. This difference appeared even more pronounced by Day 7, by which time 27% of the Control and 19% of the pOEC oocytes placed into maturation had developed to blastocysts. This apparent suppression of blastocyst growth in the pOEC group may be explained by the in vivo embryo development condition, where faster growing blastocysts secrete estrogen which inhibits the growth of late developing embryos [38,39]. Keeping in mind that the Day 7
pOEC co-cultured blastocyst rate was equal to the penetration rate (PEN), this evidence appears to corroborate the view that pOEC treatment enhances the ability of the oocyte to support and facilitate normal fertilization with the resultant development of superior quality blastocysts. Taking into account the percentage of blastocysts that had hatched, or were hatching, in the pOEC group, the progression of blastocyst growth, especially, seemed to have been accelerated in the pOEC group. This was confirmed by the average number of nuclei per blastocyst which was significantly higher for the pOEC group than for the Control group, as well as the larger proportion (of the total number) of blastocysts in pOEC group containing more nuclei. This indicated that the pOEC blastocysts were of superior quality to the Control blastocysts as forecast by the cytoplasmic index. Hatching of blastocysts is facilitated by events related to protein synthesis earlier in embryo development [15]. The pOEC treatment may thus modulate events in the ooplasm enabling the oocyte to elicit the eventual hatching of the blastocyst. The cytoplasmic index forecast the greater potential of oocytes in the pOEC group and it thus appears that the CI is more predictive of oocyte, and subsequent blastocyst quality, than the percentage pronucleus formation or normal fertilization.

In all, these results indicate that the presence of pOEC during IVM enhances the quality of cytoplasmic maturation of the oocyte, and subsequent blastocyst cell proliferation or tempo of growth. Although the percentage oocytes developing to blastocysts in the pOEC group was reduced, the blastocysts forming in this group were of superior quality as judged by their greater number of nuclei. Addition of pOEC during the second half of maturation also enhances the inherent hatchability of the resultant blastocysts within this serum-free environment. The secretions of the pOEC might provide beneficial factors such as energy substrates, growth factors [36,37] or scavenger ions and other macromolecules involved in the functioning of the ooplasm, but the precise role of pOEC in in vitro oocyte maturation and subsequent embryo development is unclear and remains intriguing.

Acknowledgements

The authors thank the Grant sponsor: EU Commission; Grant Number QLK3-CT 1999-00104, and also Mr. Ramon Cabrera for logistical support during the course of these experiments.
References