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Theriogenology 59 (2003) 975–986

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Theriogenology

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## Effects of oviductal and cumulus cells on in vitro fertilization and embryo development of porcine oocytes fertilized with epididymal spermatozoa

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Received 2 May 2002; received in revised form 30 June 2002; accepted 2 July 2002

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### Abstract

This study was designed to evaluate the effects of adding porcine oviductal epithelial cell (POEC) monolayers before or during the fertilization of denuded or cumulus-enclosed oocytes, in terms of fertilization results and subsequent embryo development. The variables determined were: penetration rate, mean number of spermatozoa per oocyte, male pronucleus formation rate, monospermy rate, cleavage rate after 48 h of fertilization, blastocyst rate, and mean number of nuclei per blastocyst. We used cumulus-free and cumulus-enclosed oocytes preincubated or fertilized in the presence of POEC, once the purity in epithelial cells of these cultures had been assessed. All the experiments involved the use of frozen-thawed epididymal spermatozoa to avoid replicate variability. The POEC cultures prepared showed a high proportion of epithelial cells (over 95%). Preincubation of oocytes with POEC before fertilization showed no effects on the fertilization variables determined. In contrast, during IVF under our experimental conditions, these cells attached to the cumulus cells and their interaction had a significant effect on some of the fertilization variables analyzed. The presence of POEC and cumulus cells during IVF increased oocyte penetrability. Moreover, in the absence of POEC, cumulus cells resulted in a reduced monospermy rate. On subsequent embryo culture, a lower cleavage and blastocyst formation rate were recorded when the oocytes had been preincubated with POEC before IVF.

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**Keywords:** Oviductal cells; Cumulus cells; Oocyte; Spermatozoa; IVF; Pig

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## 1. Introduction

For over a decade, oviductal cells have been used in culture systems in an effort to approximate laboratory conditions to those encountered by the gametes in the oviduct [19]. In recent years both oviductal cells and products or conditioned media have been employed by several research groups [5,11,13,19,28]. In pigs, the oviductal cells used are obtained from different parts of the oviduct [6,7,29], at different stages of the estrous cycle [30,32] or are introduced at different times during in vitro fertilization [28]. The effect of the presence of these cells on penetrability by ejaculated spermatozoa (fresh or frozen-thawed) has been widely investigated and, in general, they increase penetrability and according to some authors improve monospermy rates [6,19]. The use of oviductal cells before IVF has been less widely explored in spite of being shown that the preincubation of pig oocytes with oviductal cell monolayers [28] or with a porcine oviduct-specific glycoprotein [14] increases the rate of monospermic fertilization. Unfortunately, this type of study has not been developed further and the possible influence of these cells on subsequent embryo development remains to be established. Moreover, oviductal cells seem to show different behavior depending on the type of spermatozoa employed (fresh ejaculated versus cryopreserved ejaculated) possibly modifying penetrability results [28]. Although there have been reports of improved results related to the use of epididymal over ejaculated spermatozoa [25], there is scarce reference to their use in the literature. This kind of semen have some advantages in front of the ejaculated one such as its simplicity to be frozen in small containers, a higher motility and consistent in vitro fertilization rates with minimal variability [25].

Although it is widely accepted that cumulus cells are needed throughout the in vitro oocyte maturation process for appropriate cytoplasmic maturation facilitating subsequent male pronucleus formation [9,16], their behavior during IVF is yet to be established. Under in vivo conditions, porcine oocytes enter the oviduct and are fertilized while enveloped by their cumulus cells. In pig IVF systems, however, the use of cumulus-enclosed or denuded oocytes is not standardized and is usually determined by the kind of spermatozoa used: refrigerated or frozen-thawed, respectively [2,10,12,35]. The effects of these cells on penetrability by epididymal spermatozoa have not been widely investigated. Besides, these studies invariably involved stopping the process after fertilization so there is a lack of information on the subsequent effects on embryo development.

In summary, although the effects of both oviductal and cumulus cells have been examined in the past, their possible role in IVF and early embryo development and their suitability for use in IVF systems comprised of denuded or cumulus-enclosed oocytes and epididymal spermatozoa remain unclear. The present study was thus designed to evaluate the effects of porcine oviductal epithelial cell (POEC) monolayers added before and during IVF using denuded or cumulus-enclosed oocytes on fertilization results and subsequent embryo development.

## 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 [22] 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 (Foligon, 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 [26] supplemented with 3 mg/ml fatty acid-free BSA and 1.10 mmol/l Na-pyruvate. The medium for embryo culture was NCSU-23 [15] supplemented with 4 mg/ml fatty acid-free BSA and 1 mM glutamine.

Oviductal epithelial cells were cultured in TCM 199 supplemented with 13% (v/v) FCS, 150 IU/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Paisley, UK).

## 2.2. Oocyte collection and *in vitro* maturation

Ovaries from prepubertal gilts were transported to the laboratory in saline (0.9%, w/v NaCl) containing 100 mg/ml kanamycin at 37 °C. Cumulus–oocyte complexes were collected from non-atretic follicles (3–6 mm in diameter) by aspiration, and washed twice in modified Dulbecco phosphate buffered saline (PBS) supplemented with 1 mg/ml polyvinyl alcohol. Oocytes with several layers of cumulus oophorus cells showing a homogeneous and granulated cytoplasm were selected and rinsed twice in maturation medium, previously equilibrated for a minimum of 3 h under 5% CO<sub>2</sub> in air at 38.5 °C.

Groups of 50 oocytes were cultured in 500 µl maturation medium for 22 h under 5% CO<sub>2</sub> in air at 38.5 °C. After culture, the oocytes were washed three times and transferred to hormone-free maturation medium for a further 22 h [8].

## 2.3. Culture of POEC

The procedure for culture of POEC was that described by Ouhibi et al. [20] with minor modifications [28]. Oviducts from prepubertal gilts were recovered from the slaughterhouse and transported to the laboratory. They were then rinsed once in saline at 37 °C and twice in PBS before being transferred to a Petri dish within a laminar flow hood where fat pads and connective tissues were removed 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.

Following incubation, the wall of the oviduct was gently squeezed and its contents recovered in a Petri dish containing 10 ml preequilibrated culture medium. The epithelial cell clusters were dissociated by gentle, repeated pipetting followed by centrifugation at 800 × *g* for 4 min. The resultant supernatant was discarded and the pellet resuspended in fresh culture medium and seeded at a final concentration of approximately 10<sup>5</sup> cells/well. The 4-well Nunc plates were maintained at 38.5 °C under 5% CO<sub>2</sub> and the medium was changed after 48 h, and again every 2 days. Cells reached confluence after 5–7 days of initial seeding. Cell viability was directly evaluated by observing the ciliary movement and progressive growth of epithelial cells.

Cell purity was checked by indirect immunocytochemistry with a monoclonal antibody raised against cytokeratin [4,27] produced using bovine epidermal keratin as immunogen (Sigma C-6909). After 5 days of culture, the cells in the multiwell plates were washed three

times in PBS and then fixed for 7 min in methanol/acetone (7:3, v/v). Next, 50  $\mu$ l of the anti-cytokeratin antibody 8.13 diluted 1:20 (v/v) in PBS was added. The cells were incubated with the antibody for 1 h at 37 °C in a humidified chamber and then washed three times with PBS. Fifty microliters of a fluorescein isothiocyanate-labeled secondary antibody (Molecular probes, Leiden, Holland) diluted in PBS at 1:2000 (v/v) was then added followed by incubation for 1 h in a humidified atmosphere at 37 °C. The slides were rinsed three times in PBS and finally covered with 100  $\mu$ l of Hoechst 33342 in methanol 1:100 (v/v). Labeled cells were visualized using an epifluorescence microscope (magnification 400 $\times$ ) and those showing a fluorescent cytoplasm were considered to be epithelial cells.

#### 2.4. *In vitro* fertilization and embryo culture

Frozen epididymal semen from one stud boar (Large White  $\times$  Landrace) was used in all the experiments. Prior to IVF, three 0.25 ml straws were thawed in warm water (20 s at 38 °C). After estimating sperm motility under a phase contrast microscope (magnification 200 $\times$ ), the semen samples were diluted in Androhep<sup>®</sup> (Minitüb, Tiefenbach, Germany) and centrifuged at 800  $\times$  g for 3 min. The sperm pellet was resuspended in modified TALP medium equilibrated overnight in an incubator at 38.5 °C under 5% CO<sub>2</sub>. In all the experiments, the final sperm concentration was adjusted to a ratio of 1500 spermatozoa:oocyte, and 10  $\mu$ l of diluted spermatozoa were added to the plates containing the oocytes equilibrated for 30 min before introducing the sperm.

After an IVF period of 18 h, presumptive zygotes were cultured in groups of 40 maximum for an additional 7 days in 2 ml NCSU-23 medium [23]. The medium was replaced once after 2 days of culture.

#### 2.5. *Assessment of fertilization results and developmental capacity of embryos*

After the IVF period, a representative sample of each group was fixed in acetic acid–ethanol (1:3, v/v), stained with 1% (w/v) lacmoid after a minimum of 24 h, and examined under a phase contrast microscope (magnification 400 $\times$ ) for evidence of sperm penetration and pronucleus formation.

Forty-eight hours after insemination, the cleavage of oocytes was evaluated under a stereomicroscope equipped with a warm plate at 38.5 °C and a special chamber connected to a 5% CO<sub>2</sub> permanent supply tube. Two 4-cell stage embryos were recorded as cleaved. Blastocysts were assessed on day 7 by observation of a clear blastocoele under the stereomicroscope. Nuclei were counted by fixing and staining with Hoechst 33342 1% in PBS.

#### 2.6. *Experimental design*

Before the *in vitro* use of POEC, we conducted a pilot experiment to test the purity of the epithelial cell cultures. Oviductal cells were cultured as previously described and subjected to four replicate immunocytochemical tests [4].

In the Experiment 1, we investigated the effects of preincubating denuded and cumulus-enclosed oocytes with POEC before IVF on fertilization results and embryo development. After the IVM period, half of a group of oocytes was preincubated for 3 h with oviductal

epithelial cell monolayers pre-equilibrated for 1 h in modified TALP medium (POEC preincubation group) and the other half was maintained in maturation medium (non-preincubated oocyte group). The oocytes used were cumulus-enclosed or were denuded by pipetting. After the 3 h, the non-preincubated oocytes were denuded or not and then all the oocytes were fertilized in the absence of oviductal cells. Oocytes were transferred to 90  $\mu$ l of TALP medium covered with paraffin oil and after 30 min at 38.5 °C and 5% CO<sub>2</sub>, 10  $\mu$ l of sperm solution was introduced. After IVF, a subsample of 15 oocytes from each group was fixed and stained to assess the fertilization results, and the remaining zygotes were transferred to embryo culture. Cleaved embryos, blastocyst formation, and number of nuclei per blastocyst were recorded 2 and 7 days after fertilization, respectively. This experiment was performed as six replicates and the raw data were pooled.

Experiment 2 was designed to evaluate the effects of adding POEC during the IVF of cumulus-free and cumulus-enclosed oocytes on fertilization and embryo development variables. After IVM, half of the oocytes were denuded by pipetting and the other left intact with their cumulus cells. These oocytes were then washed twice in modified TALP medium, transferred to a 4-well plate and fertilized in the presence (POEC IVF group) or absence (POEC-free IVF group) of a monolayer of oviductal cells equilibrated for 1 h in 190  $\mu$ l of TALP. After the IVF period, a subsample of 15 oocytes from each group was fixed and stained to assess the fertilization results and the remaining zygotes were transferred to an embryo culture medium. Cleaved embryos and the rate of blastocyst formation and number of nuclei per blastocyst were recorded 2 and 7 days after fertilization, respectively. This experiment was performed as five replicates and raw data were pooled.

### 2.7. Statistical analysis

Data are presented as the mean  $\pm$  S.E.M., and all rates were modeled according to the binomial model of parameters. The variables, oocyte penetration rate, number of sperm cells per penetrated oocyte, male pronucleus formation and monospermy rates, as well as cleavage and blastocyst formation rates, and the number of cells/blastocyst were analyzed by two-way ANOVA, considering the presence of POEC and cumulus cells as 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

The immunocytochemistry test confirmed that  $99.30 \pm 1.85\%$  of the oviductal cells grown *in vitro* were epithelial, showing a positive staining with the anti-cytokeratin 8.13 antibody (Fig. 1).

### 3.1. Experiment 1

Preincubating the oocytes with oviductal epithelial cells for 3 h before IVF showed no effect on any of the fertilization variables determined (Table 1). Cumulus-enclosed oocytes tended to be more penetrated than denuded ones (*P* = 0.06) although if we focus

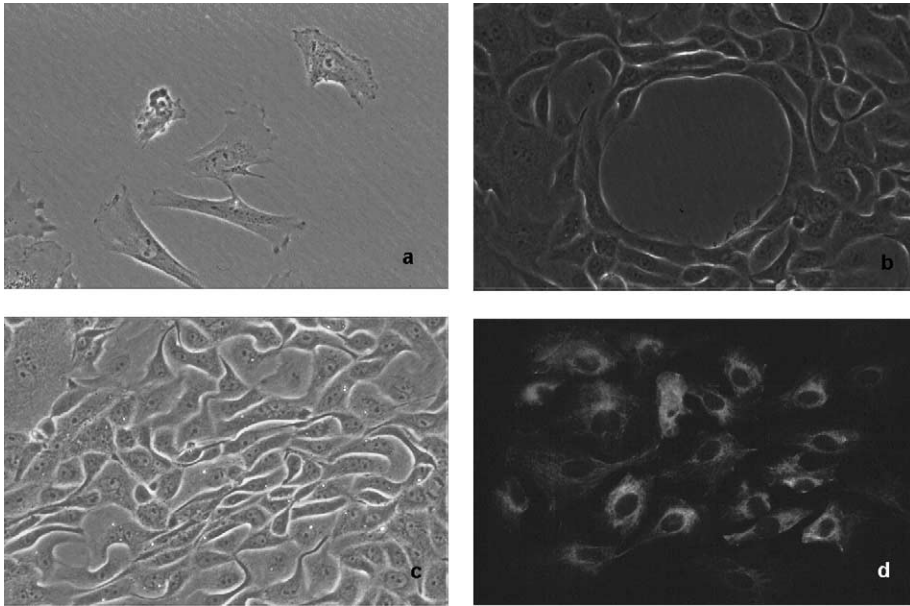


Fig. 1. (a) Isolated oviductal cells attached to the bottom of the Petri dish after 72 h of culture. (b) Oviductal cells after 5 days of culture showing a non-covered area. (c) Confluent monolayer of POECs after 7 days of culture. (d) POECs. Immunostaining with anti-cytokeratine 8.13 showing the positive staining of the cytoplasm.

exclusively on the group of POEC preincubated oocytes, penetration rates were similar for the denuded oocytes and those surrounded by their cumulus cells (93.51 versus 92.22). The presence of cumulus cells led to a significantly increased mean number of sperm per oocyte, both in the POEC preincubated oocyte and in the non-preincubated control groups. However, no effect of cumulus cells was observed on male pronucleus formation after IVF, which was over 90% in all the groups.

Table 1  
Effect of preincubating oocytes with POEC on the fertilization of cumulus-enclosed and denuded oocytes

POEC preincubation	Cumulus	N	Penetration (%)	S/O	% MPN <sup>1</sup>	Monospermy (%) <sup>1</sup>
No	No	83	83.13 ± 4.14 <sup>a</sup>	2.10 ± 0.14 <sup>a</sup>	95.65 ± 2.47	43.48 ± 6.01 <sup>a</sup>
	Yes	81	96.30 ± 2.11 <sup>b</sup>	4.50 ± 0.24 <sup>b</sup>	98.72 ± 1.28	6.41 ± 2.79 <sup>b</sup>
Yes	No	77	93.51 ± 2.83 <sup>ab</sup>	2.51 ± 0.17 <sup>a</sup>	91.67 ± 3.28	25.00 ± 5.14 <sup>c</sup>
	Yes	77	92.22 ± 3.07 <sup>ab</sup>	4.08 ± 0.26 <sup>b</sup>	97.18 ± 1.98	11.27 ± 3.78 <sup>bc</sup>
Source of variability						
POEC preincubation			0.319	0.994	0.238	0.132
Cumulus			0.060	<0.001	0.067	<0.001
POEC preincubation × cumulus			0.022	0.056	0.600	0.010

N, number of oocytes; S/O, mean number of spermatozoa per penetrated oocyte; and % MPN, male pronucleus formation rate. Values with different superscripts (a, b, c) within columns are significantly different (P < 0.05).

<sup>1</sup> With respect to the number of penetrated oocytes.

Table 2

Effect of preincubating oocytes with POEC before IVF on embryo development of cumulus-enclosed and denuded oocytes

POEC preincubation	Cumulus	<i>N</i>	Cleavage (%)	Blastocysts (%) <sup>1</sup>	<i>N</i> cells/blastocyst
No	No	360	80.56 ± 2.09	30.69 ± 2.71 <sup>a</sup>	20.47 ± 1.17
	Yes	383	80.42 ± 2.03	21.43 ± 2.34 <sup>b</sup>	18.17 ± 1.26
Yes	No	317	74.13 ± 2.46	17.45 ± 2.48 <sup>b</sup>	20.04 ± 1.49
	Yes	322	76.40 ± 2.37	17.07 ± 2.40 <sup>b</sup>	19.47 ± 2.01
Source of variability					
POEC preincubation			0.019	<0.001	0.784
Cumulus			0.633	0.056	0.366
POEC preincubation × cumulus			0.590	0.078	0.583

*N* is the number of zygotes. Values with different superscripts (a, b) within columns are significantly different ( $P < 0.05$ ).

<sup>1</sup> With respect to the number of cleaved zygotes.

In contrast, the presence of cumulus cells had a detrimental effect on monospermy rates ( $P < 0.001$ ), best results being achieved with denuded oocytes. This effect lost its significance, however, when the oocytes were preincubated with oviductal cells before IVF (25 versus 11.27).

The embryo development results (Table 2) indicate that embryos formed from non-preincubated oocytes showed a significantly higher cleavage rate than those derived from POEC preincubated oocytes ( $P = 0.019$ ). The presence of cumulus cells showed no effect on this variable. Preincubation with POEC had a negative effect on the blastocyst formation rate ( $P < 0.001$ ) and this trend was also related to the presence of cumulus cells ( $P = 0.056$ ). Thus, highest blastocyst formation rates were achieved after the IVF of non-POEC preincubated, denuded oocytes.

Finally, neither POEC preincubation nor cumulus cell presence had an effect on the final quality of the blastocyst, assessed by the mean number of cells per blastocyst, which was similar in each group.

### 3.2. Experiment 2

The presence of a monolayer of oviductal cells during the fertilization of oocytes with epididymal spermatozoa significantly increased penetrability (Table 3), both in terms of the penetration rate ( $P = 0.010$ ) and the mean number of sperm per oocyte ( $P < 0.001$ ).

Cumulus-enclosed oocytes showed a tendency towards easier penetrability than denuded oocytes ( $P = 0.054$ ). A significant effect on the mean number of spermatozoa was observed ( $P = 0.027$ ), highest values being recorded for oocytes fertilized in the presence of both types of cells (3.29 spermatozoa/oocyte). The latter was significantly different from that noted for oocytes fertilized in the absence of POEC.

As in Experiment 1, neither the presence of oviductal or cumulus cells during IVF affected male pronucleus formation, which again was around 90% in each group. Similarly, no effects of either cell type were observed on the final rates of monospermy, which ranged

Table 3

Effect of POEC monolayers on the fertilization of cumulus-enclosed and denuded oocytes

POEC IVF	Cumulus	<i>N</i>	Penetration (%)	<i>S/O</i>	% MPN <sup>1</sup>	Monospermy (%) <sup>1</sup>
No	No	73	76.71 ± 4.98 <sup>a</sup>	2.12 ± 0.19 <sup>a</sup>	89.29 ± 4.17	35.71 ± 6.46
	Yes	70	88.57 ± 3.83 <sup>ab</sup>	2.33 ± 0.15 <sup>a</sup>	93.55 ± 3.15	30.65 ± 5.90
Yes	No	78	91.03 ± 3.26 <sup>b</sup>	2.59 ± 0.20 <sup>ab</sup>	91.55 ± 3.32	30.99 ± 5.53
	Yes	79	93.67 ± 2.76 <sup>b</sup>	3.29 ± 0.24 <sup>b</sup>	93.24 ± 2.94	21.62 ± 4.82
Source of variability						
POEC			0.010	<0.001	0.772	0.225
Cumulus			0.054	0.027	0.379	0.202
POEC × cumulus			0.220	0.237	0.704	0.704

*N*, number of oocytes; *S/O*, mean number of spermatozoa per penetrated oocyte; and % MPN, male pronucleus formation rate. Values with different superscripts (a, b) within columns are significantly different ( $P < 0.05$ ).

<sup>1</sup> With respect to the number of penetrated oocytes.

Table 4

Effect of POEC monolayers during the IVF of cumulus-enclosed and denuded oocytes on embryo development

POEC IVF	Cumulus	<i>N</i>	% Cleavage	Blastocysts (%) <sup>1</sup>	<i>N</i> cells/blastocyst
No	No	320	59.37 ± 2.75 <sup>a</sup>	21.58 ± 2.99	22.65 ± 1.98
	Yes	305	71.48 ± 2.59 <sup>b</sup>	17.89 ± 2.60	19.00 ± 1.21
Yes	No	349	68.48 ± 2.49 <sup>ab</sup>	14.64 ± 2.29	20.00 ± 1.74
	Yes	288	71.87 ± 2.65 <sup>b</sup>	16.91 ± 2.61	16.94 ± 1.15
Source of variability					
POEC			0.070	0.130	0.160
Cumulus			0.003	0.785	0.046
POEC × cumulus			0.098	0.255	0.858

*N* is the number of zygotes. Values with different superscripts (a, b) within columns are significantly different ( $P < 0.05$ ).

<sup>1</sup> With respect to the number of cleaved zygotes.

from 21.62 to 35.71%. This last value corresponds to oocytes fertilized in the absence of both oviductal and cumulus cells.

The embryo culture data (Table 4) indicate that the presence or absence of oviductal cells during IVF failed to affect any of the embryo development variables examined, although there was a tendency towards higher cleavage rates ( $P = 0.07$ ) when these cells were present. In contrast, cumulus cells showed a positive effect on cleavage rate ( $P = 0.003$ ) but not on the blastocyst formation rate.

#### 4. Discussion

The immunocytochemical detection of keratins is the most commonly used method of characterizing epithelial cell cultures [4,27]. In our study, this technique served to confirm

the purity of our oviductal cell cultures in epithelial cells (over 95%) and the lack of contamination with other cell types. These findings are comparable to those reported by others for bovine cell cultures [27,31,33].

The use of oviductal cells for oocyte preincubation before IVF and during IVF using ejaculated spermatozoa [11,19] has been associated with improved rates of monospermy. In the present study, the preincubation of oocytes with POEC for 3 h before IVF showed no effect on the fertilization variables recorded (Table 1). Possible explanations for this could be an insufficiently long preincubation time to considerably affect the oocyte, the incapacity of the IVF system to discriminate possible effects (given the high spermatozoa:oocyte ratio) or that the POEC culture employed was not “functional” or “active.” In our opinion, any of these three hypotheses might be valid. Indeed, beneficial effects on monospermy have been described after the preincubation of porcine oocytes with POEC [28] or purified porcine glycoproteins [14]. In both these studies, the contact time ranged from 2 to 4 h before IVF and ejaculated spermatozoa were used. Some authors propose that the effect of the oviductal cell disappears or is masked when high sperm concentrations are used [5,28]. Finally, with regard to the functionality of the culture, it has been shown that oviductal cells treated with estradiol give rise to improved monospermy rates over non-treated or progesterone-treated cells [6]. In our study, we were able to confirm that the oviductal cell cultures used were mainly epithelial but we have no information on their “functionality.” The fact that preincubation with POEC also failed to affect the rate of male pronucleus formation is in agreement with the observations of other researchers working with preincubated oocytes [14,28] suggesting that the cytoplasmic maturation of oocytes is sufficient to achieve a high rate of pronucleus formation after IVM in NCSU-37.

Cumulus cells have been described to show a positive effect on penetrability by ejaculated frozen-thawed spermatozoa [35,36] and were associated here with the penetration of the oocyte by a greater number of epididymal spermatozoa. For different species [3,17] several authors have referred to a beneficial effect of the cumulus on the acrosome reaction and this could account for the higher penetrability of the cumulus-enclosed oocytes we observed. Nevertheless, this enhanced penetrability led to a diminished monospermy that was significant in the case of the oocytes not preincubated with POEC. Frozen-thawed spermatozoa show reduced motility after thawing but those able to cross the mass of cumulus cells before completely losing their motility can maintain their activity and undergo capacitation and subsequently penetrate the oocyte [34]. According to our results, this effect of cumulus cells also appears to apply to epididymal spermatozoa and is in line with previous findings [12]. The dependence on cumulus cells of frozen boar epididymal spermatozoa to complete capacitation was suggested by Nagai et al. [18]. However, the results of both our experiments indicate that with the system employed, high penetration rates of denuded oocytes can also be achieved using epididymal spermatozoa.

It should be noted that the oocytes preincubated with POEC before IVF showed similar penetration rates, irrespective of the presence or absence of cumulus cells (93.51 versus 92.22, Table 1). This could be attributable to some kind of interaction between these two cell types leading to alterations at a level (e.g. the zona pellucida) not reflected by conventional fertilization variables.

As some authors have already indicated, cleavage rate does not objectively assess the embryo culture system [1]. Moreover, a high proportion of in vitro produced pig embryos have some kind of morphological abnormality and a low number of cells [37]. Here, as a logical consequence of the rate of monospermy, according to the blastocyst formation rate, non-POEC preincubated, denuded oocytes showed the highest output, although the mean number of cells/blastocyst was similar between groups and within the range quoted by other authors [24,26].

In the second experiment, oocytes subjected to IVF in the presence of POEC showed increased penetrability (Table 3). These same observations were previously associated with the use of ejaculated spermatozoa, both refrigerated [11,19] and frozen-thawed [21,28] but, to our knowledge, our results are the first to refer to the use of epididymal spermatozoa. This observation could be interpreted as the effect of the oviductal cells on the spermatozoa favoring sperm capacitation, since in this experiment, unlike the previous one, the monolayers were present for the entire 18 h of contact between gametes. These data would be in agreement with those reported by Fazeli et al. [7], who observed sperm capacitation induced by oviductal cells in boar ejaculated spermatozoa. However, the presence of POEC during IVF did not significantly affect male pronucleus formation (Table 3) in agreement with other papers [11,19]. As in Experiment 1, although perhaps not as evident, cumulus-enclosed oocytes presented a higher number of sperm per oocyte than their denuded counterparts. These data would also appear to point to a beneficial effect of cumulus and oviductal cells on sperm capacitation and the acrosome reaction or, at least, on oocyte penetrability.

The effects observed on the variables of fertilization outcome could not be extrapolated to subsequent embryo development since no effect was shown by the presence of POEC during IVF on the cleavage and blastocyst rates or mean number of cells per embryo (Table 4). Although in this experiment the presence of cumulus cells during IVF did have a significant effect on the cleavage rate, in our opinion we should be cautious in our interpretation because of the subjectivity involved in evaluating this variable as mentioned previously. The proof of this is that the rate of blastocyst formation showed no effect of the cumulus and is, no doubt, a consequence of the cleavage rate.

In conclusion, our data show that the preincubation of oocytes with POECs 3 h before IVF does not affect the typically estimated fertilization variables and that the copresence of these and cumulus cells during IVF with epididymal spermatozoa increases oocyte penetrability. Finally, under the experimental conditions employed neither POEC preincubation nor presence of POEC during IVF improved the embryo development results.

## Acknowledgements

The authors would like to thank Dr. Wilfrid Kues (Institute of Animal Science and Animal Husbandry, Mariensee, Germany) for his experienced assistance in the immunocytochemistry test, and Mrs. Antje Frenzel and Mrs. Birgit Sieg for their valuable help in the laboratory. This research was supported by projects 1FD97-501, HA99-115 (DAAD 314), and AGL 2000-0485-C02-01.

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