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Effect of co-culture of porcine sperm and oocytes with porcine oviductal epithelial cells on in vitro fertilization

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Abstract

This study was designed to determine the effect of co-culture with porcine oviductal epithelial cell (POEC) monolayers on in vitro fertilization of pig oocytes. The in vitro penetrability of mature (experiment 1) or immature (experiment 2) oocytes was studied in presence or absence of POEC during IVF with fresh semen. In experiment 3, boar and POEC effects were analyzed but in this case with frozen-thawed spermatozoa. In experiment 4, the spermatozoa were pre-incubated before IVF with or without POEC in order to assess their effect on IVF sperm-related parameters. In experiment 5, the effect of POEC was studied by co-culturing them with oocytes before IVF to determine if monospermy was improved. The results showed that high sperm concentration and POEC increase oocyte penetrability ($P < 0.01$) and decrease monospermy rate ($P < 0.01$), in both mature and immature oocytes ($P < 0.01$) with fresh semen and a 18 h culture time. With frozen semen was detected a boar and POEC effect ($P < 0.01$) on penetration rate. The sperm pre-culture 2 h with POEC also resulted in an increase of sperm penetration in terms of number of sperm per oocyte ($P < 0.01$) and this treatment did not increase monospermy when contact time between gametes was limited to 6 h although monospermy was higher when POEC were present during IVF. Finally, exposure of oocytes to POEC for 4 h before IVF facilitated monospermic penetration to over 70% ($P < 0.01$). In conclusion, the use of POEC in porcine IVF systems provides the possibility of working with low sperm concentrations and the effect of POEC on monospermy depends on sperm concentration, boar and contact time between gametes. Moreover, the exposure of oocytes to POEC before IVF improves the rate of monospermy.

Keywords: Oviductal cells; Oocyte; Spermatozoa; IVF; Pig-reproductive technology

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1. Introduction

In mammals, the oviduct provides the appropriate microenvironment for important reproductive processes like gamete transport, fertilization and early embryonic development. This microenvironment is achieved by the inner surface of the oviduct as well as the oviductal fluid. An increasing number of studies on this subject have been reported and various growth factors and receptors have been identified in the oviducts from some mammalian species (Gabler et al., 1997; Wollenhaupt et al., 1997). In spite of the fact that some of the oviductal factors that affect these important reproductive processes and their precise role are still unknown (Buhi et al., 1997; Smith and Nothnick, 1997; Smith, 1998), oviductal cells can be used both to investigate sperm–oviduct interactions and to recreate the appropriate conditions for a normal fertilization approximating the IVF process to in vivo conditions. These are some of the reasons why oviductal epithelial cell cultures have recently been employed by several research groups specially in bovine (Chian and Sirard, 1995; Katska et al., 1995; McNutt-Scott and Harris, 1998; Pollard et al., 1991; Rieger et al., 1995). In pigs these kind of cells have been employed both before and after in vitro fertilization (Dubuc and Sirard, 1995; Kano et al., 1994; Martínez et al., 1993) and under different conditions: vesicles in suspension (Dubuc and Sirard, 1996; Park and Sirard, 1996), monolayers (Dubuc and Sirard, 1996; Gadea et al., 1998; Kano et al., 1994; Nagai and Moor, 1990), cells from different parts of the oviduct (Dubuc and Sirard, 1996; Fazeli et al., 1999; Suarez et al., 1991), from different stages of the cycle (Techakumphu and Srianan, 1994; Vatzias and Hagen, 1999) and even oviductal cell-conditioned medium (Kano et al., 1994; Vatzias and Hagen, 1999).

In porcine, these in vitro studies have shown that oviductal cells have a beneficial effect on fertilization of mature oocytes inseminated with fresh semen (Dubuc and Sirard, 1995; Kano et al., 1994; Nagai and Moor, 1990), but we have no references employing immature oocytes, even though these oocytes have been used in pig IVF assays (Martínez et al., 1993). It is still unknown if oviductal cells are able to exert some effect regardless of the maturational stage of the oocytes or the degree of maturation of the zona pellucida. With reference to spermatozoa, both fresh and frozen-thawed spermatozoa can penetrate oocytes in vitro, but their conditioning by oviductal cells influences the rate of polyspermy (Gadea et al., 1998; Park and Sirard, 1996). Besides, a boar effect on the different IVF parameters is expected, in a similar way as occurs when fresh semen is employed (Coy et al., 1999; Xu et al., 1996).

In vivo, polyspermy is controlled in part by the regulatory role of the oviduct (Hunter, 1991). In farm animals, a population of viable spermatozoa enters the oviducts a few hours after mating and the cells are arrested in the caudal part of the isthmus for 18 h or more before the moment of ovulation. Then they are released towards the site of fertilization, at the ampullary isthmic junction, apparently showing a greatly enhanced form of motility, the so-called hyperactivated or whiplash condition (Hunter, 1984). Contact between sperm and the oviductal cells is known to be beneficial for in vitro sperm capacitation and survival in different species (Dubuc and Sirard, 1996; Gutiérrez et al., 1993; Pollard et al., 1991; Sidhu et al., 1998; Smith and Nothnick, 1997). In pigs, this sperm–oviductal cell contact constitutes a final phase of maturation that gives spermatozoa the ability to penetrate the egg investments (Hunter, 1984). In addition some authors have pointed out that oocyte

maturation is completed in the oviduct (Hunter, 1989; Sidhu et al., 1998), where it could be attached to oviductal factors. In this way, oocytes would be more competent to the fertilization process. The in vitro exposure of bovine gametes to an oviduct specific glycoprotein improves the fertilization rates (Martus et al., 1998). Although porcine spermatozoa have already been pre-cultured with oviductal cells with good results on monospermy (Dubuc and Sirard, 1995; Nagai and Moor, 1990), to our knowledge there are no data about oocyte pre-incubation with oviductal cells.

This paper was carried out to study the effect of porcine oviductal epithelial cell (POEC) monolayers on both porcine gametes during different phases of the IVF process. The objectives were: (1) to study the effect of POEC on in vitro fertilization results of mature oocytes at different sperm concentrations, (2) to investigate if these cells have some effect on immature oocytes, (3) to evaluate the effect of POEC and boar on in vitro fertilization with frozen-thawed sperm, (4) to assess the effect of POEC during sperm pre-culture before in vitro fertilization, and (5) to determine the effect of POEC monolayers on the oocytes which are pre-incubated before in vitro fertilization.

2. Material and methods

2.1. Culture media

Unless it is indicated, all chemicals used in this study were purchased from Sigma-Aldrich Química SA (Madrid, Spain). The medium used for oocyte maturation was Waymouth MB 725/1 supplemented as it was previously described (Coy et al., 1999; Yoshida et al., 1992) with 10 IU/ml eCG (Foligon, Intervet International BV, Boxmeer, Holland), 10 IU/ml hCG (Chorulon, Intervet International BV, Boxmeer, Holland), 1 μg/ml estradiol-17β, 10% (v/v) fetal calf serum (Biological Industries, Beitz Haemek, Israel) and 10% porcine follicular fluid (v/v).

The fertilization medium (199-IVF) consisted of TCM 199 supplemented with 0.91 mM Na-pyruvate, 8.75 mM Ca-lactate, 3.05 mM glucose, 3.6 mM caffeine, 50 IU/ml penicillin G and 30 μg/ml streptomycin sulfate.

The culture medium for POEC (199-POEC) consisted of TCM 199 supplemented with 13% FCS, 150 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Paisley, UK).

2.2. Oocyte collection and in vitro maturation

Within 30 min of slaughter, ovaries from commercial prepubertal gilts (landrace × large white) were transported to the laboratory in saline (0.9% w/v NaCl) containing 100 μg/ml kanamycin and 50 μg/ml polymyxin B sulfate at 37°C and washed once in 0.04% cetrimide solution and twice in saline. Oocytes-cumulus cell complexes collected from nonatretic follicles (3–6 mm diameter), were pooled, washed twice in 35 mm plastic Petri dishes containing Dulbecco's phosphate buffered saline modified supplemented with 4 mg/ml PVA (PBSDm) and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO_2 in air. After 20–22 h of culture the oocytes were transferred

to fresh maturation medium without hormonal supplements, washed twice and cultured for an additional 20–22 h (Funahashi and Day, 1993).

2.3. Cryopreservation of boar semen

The method of semen cryopreservation was essentially the same as that previously described (Almild and Johnson, 1988; Westendorf et al., 1975) with slight modifications. Ejaculates from 2–3-year-old stud boars were diluted with Beltsville thawing solution (BTS; Pursel and Johnson, 1975), at a ratio of 1:1, placed at 15°C for 2 h and later centrifuged (800 × g, 10 min). The supernatant was discarded and the pellet was resuspended with lactose-egg yolk extender (LEY, 80 ml of 11% lactose and 20 ml egg yolk) to provide 1.5×10^9 cells/ml. After further cooling to 5°C over a 90 min period, two parts of LEY-extender semen were mixed with LEY extender plus 1.5% Orvus Es Paste (Minitüb, Tiefenbach, Germany) and 9% glycerol. The final concentration of spermatozoa to be frozen was 1×10^9 cells/ml and glycerol 3%. The diluted and cooled semen was loaded into 0.5 ml straws (Minitüb, Tiefenbach, Germany), placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min and the straws were then stored until thawing.

2.4. In vitro fertilization

After 40–44 h of culture, the oocytes were washed twice in the fertilization medium and groups of 20 oocytes were placed into Petri dishes containing 2 ml of this medium, previously equilibrated at 38.5°C under 5% CO_2. In experiment 2, groups of 20 immature oocytes, collected just 1 h before, were also introduced into the fertilization dishes under the same conditions as mature oocytes.

For insemination with fresh semen, the sperm was prepared as previously described (Martinez et al., 1996). The sperm rich fractions were collected by the gloved hand method from 2–3-year-old stud boars and diluted at 3×10^7 cells/ml in BTS extender. After 24 h, sperm samples were washed by centrifugation (120 × g, 3 min) in order to remove heavy particles. Supernatant was concentrated (1250 × g, 3 min) and the pellet was resuspended in medium 199 to the desired final concentration for each experiment. A final volume of 100 μl was always introduced into Petri dishes containing the oocytes, except in experiment 4.

For insemination with frozen semen, a ministraw for each boar was thawed by immersing the straws in a circulating water bath at 50°C for 12 s. Immediately after thawing, the semen was diluted in BTS and subsequent methodology was the same as with fresh semen to the desired final concentration.

After the culture period, oocytes were fixed, stained with 1% (w/v) lacmoid and examined at 400× magnification for evidence of sperm penetration and pronuclear formation under a phase contrast microscope (Coy et al., 1999).

2.5. Culture of porcine oviductal epithelial cells

The procedure for culture of POEC was basically that described by Ouhibi et al. (1991) with minor modifications. Oviducts from commercial prepubertal gilts (landrace × large white) were recovered from the slaughterhouse and transported to the laboratory in saline at

37°C. They were then rinsed once in 0.04% cetrimide solution and twice in PBSDm before being transferred to a Petri dish within a laminar flow hood. 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, immersed in a Petri dish containing fresh solution and incubated at 38.5°C for 45 min.

The cells used for culture were collected in a Petri dish after a second flush with fresh Trypsin-EDTA solution. To facilitate the dissociation, the epithelial cell clusters were dissociated by a gentle, repeated pippeting. The cells were then introduced into 10 ml graduate conical tubes and 199-POEC medium (containing fetal calf serum as indicated above) was added in order to inactivate Trypsin. After centrifugation at 800 × *g* for 4 min, the supernatant was discarded and the pellet resuspended in fresh 199-POEC medium at a final concentration of approximately 10^7 cells/ml. Then, 100 μl of this solution were introduced into Petri dishes previously equilibrated 2 h at 38.5°C under 5% CO_2 containing 1900 μl of fresh 199-POEC medium. The culture dishes were maintained in these conditions and the medium was changed after 48 h and again every 2 days. Cell viability was evaluated in a direct way by observing the cilia movement of epithelial cells as well as the progressive growth. Cells reached confluence after 7–9 days of initial seeding.

2.6. Experimental design and statistical analysis

Experiment 1 investigated the effect of POEC and sperm concentration on IVF results with mature oocytes. In vitro matured oocytes were fertilized in presence (+) or absence (−) of POEC monolayers with fresh semen at different sperm concentrations: 1×10^4, 5×10^4, 1×10^5 and 5×10^5 cells/ml.

Experiment 2 studied the effect of POEC and sperm concentration on IVF results with immature oocytes. The oocytes were collected and fertilized with fresh sperm in presence (+) or absence (−) of POEC. In this experiment, sperm concentrations were 5×10^5, 1×10^6 and 5×10^6 cells/ml.

Experiment 3 tried to find out the effect of POEC and boar on IVF results with frozen-thawed semen. In vitro matured oocytes were fertilized with frozen-thawed semen from two different boars (A and B). As in previous experiments, oocytes were fertilized in presence (+) or absence (−) of POEC and the sperm concentration for IVF was 10^7 cells/ml for both boars.

Experiment 4 tested the effect of spermatozoa POEC co-culture before IVF on the sperm-related parameters. Pig oocytes matured in vitro were fertilized in presence (+) of POEC with spermatozoa previously exposed to POEC for different periods: 2 h (2 h POEC group) or 0 h (0 h POEC group). In this experiment 1×10^5 cells/ml were placed into Petri dishes with a monolayer of POEC for 2 h and after this time 75–80% of spermatozoa attached to the cells. IVF medium with non-attached spermatozoa was removed with a 2 ml pipette and exact sperm concentration in this solution was calculated. The final number of spermatozoa/ml attached to POEC in the original Petri dishes was calculated by subtracting, and this was the final sperm concentration used for 0 h POEC group. Oocytes from the control group were fertilized at the same sperm concentration with no exposed spermatozoa and in absence (−) of POEC.

A parallel group of oocytes was fertilized in presence of POEC with spermatozoa pre-incubated 2 h in IVF medium without POEC at the same concentration of 2 h POEC, 0 h POEC and control groups.

This experiment was carried out with a low and a high penetrability boar selected from previous results both with similar motility rates.

Experiment 5 investigated the effect of oocyte exposure to POEC before IVF. In vitro fertilization was performed in presence (+) of POEC with pig oocytes matured in vitro and previously exposed to POEC for different periods: 4 h (4 h POEC group), 2 h (2 h POEC group) or 0 h (0 h POEC group). The control group consisted of non-exposed oocytes fertilized in absence (−) of POEC. For all groups sperm concentration was 1×10^5 cells/ml.

2.6.1. Coincubation time and replicates

In experiments 1, 2 and 3 coincubation time was 18 h, and 6 h in experiments 4 and 5 where after removing the excess of spermatozoa attached to the zona pellucida by pipetting, oocytes were transferred to fresh IVF medium without oviductal cells to complete the entire culture period of 18 h.

Every day oocytes for all the experimental groups were recovered (at least 20 oocytes per group). Three to five replicates in different days were done for each experiment and raw data were pooled.

2.6.2. Statistical analysis

Data are presented as mean ± S.E.M. Data for all rates were modeled according to the binomial model of parameters. The rate of oocyte penetration, number of sperm cells per penetrated oocyte, male pronucleus formation and monospermy rates were analyzed by two-way ANOVA: considering the sperm concentration and POEC in experiments 1 and 2 as well as boar and POEC in experiment 3 as main effects. Results from experiments 4 and 5 were analyzed by one-way ANOVA. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered statistically different at $P < 0.05$.

3. Results

3.1. Effect of porcine epithelial cell monolayers and sperm concentration on fertilization of in vitro matured oocytes

Both sperm concentration and oviductal cells during IVF had an influence on the penetration rate ($P < 0.01$), number of spermatozoa per oocyte ($P < 0.01$) and rate of monospermy ($P < 0.01$; Table 1). Although rate of penetration decreased as a lower concentration was used, when IVF was performed in presence of oviductal cells, acceptable results were achieved even with the lowest sperm concentration (91.67 versus 34.92 at 10^4 cells/ml). Moreover, a greater number of spermatozoa per penetrated oocyte were observed with the highest concentrations, and this effect was more accentuated in presence of POEC.

With regard to monospermy rate, it was higher as sperm concentration decreased and the harmful effect of oviduct epithelial cells achieved its maximum when extreme concentrations

Table 1
IVF results for mature oocytes fertilized with different sperm concentrations (cells/ml) without (−) or with (+) POEC[a,b]

Cells/ml	POEC	*n*	%PEN	S/O	%MPN[c]	%MON[c]
1×10^4	−	63	34.92 ± 6.05 a	1.40 ± 0.15 ab	54.55 ± 10.87 abc	68.18 ± 10.16 a
	+	60	91.67 ± 3.60 bc	1.85 ± 0.16 a	47.27 ± 6.79 a	54.55 ± 6.78 a
5×10^4	−	100	76.00 ± 4.29 bd	2.45 ± 0.41 ab	59.21 ± 5.67 ac	56.58 ± 5.72 a
	+	114	71.05 ± 4.27 d	4.53 ± 0.51 b	61.73 ± 5.43 abc	32.10 ± 5.22 bc
1×10^5	−	104	70.19 ± 4.51 d	2.26 ± 0.20 a	84.51 ± 4.32 b	40.85 ± 5.88 ab
	+	131	93.89 ± 2.10 c	6.60 ± 0.51 c	71.54 ± 4.10 bc	19.51 ± 3.60 cd
5×10^5	−	93	98.92 ± 1.08 c	6.91 ± 0.42 c	70.65 ± 4.77 abc	8.70 ± 2.95 de
	+	110	100 c	11.71 ± 0.58 d	60.91 ± 4.67 ac	0.91 ± 0.91 e
Source of variability						
Cells/ml			<0.001	<0.001	<0.001	<0.001
POEC			<0.001	<0.001	0.106	<0.001
Cells/ml by POEC			<0.001	0.001	0.472	0.199

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), rate of male pronuclear formation (%MPN) and monospermy rate (%MON).
[b] Different letters in the same column indicate significantly different values ($P < 0.05$).
[c] From the penetrated oocytes.

were used: 0.91% of monospermy at 5×10^5 cells/ml. Besides, at intermediate sperm concentrations, monospermy rate was significantly higher in oocytes fertilized without oviductal cells. Concerning male pronuclear formation, no differences were found at the same sperm concentration, although a global effect was observed. Presence or absence of POEC during IVF did not have not any effect on this parameter ($P = 0.106$).

3.2. Effects of porcine epithelial cell monolayers and sperm concentration on fertilization of immature oocytes

An effect of sperm concentration and POEC was observed on all parameters studied: penetration rate ($P < 0.01$), number of spermatozoa per oocyte ($P < 0.01$) and monospermy rate ($P < 0.01$; Table 2). Again penetration rate and number of spermatozoa per penetrated oocyte increased along with sperm concentration. In all groups, penetration was significantly higher when oviductal cells were present during the in vitro fertilization process. Only at 5×10^6 cells/ml group monospermy rate was significantly different, being higher in those oocytes fertilized in absence of POEC.

3.3. Effect of boar and porcine epithelial cell monolayers on fertilization of in vitro matured oocytes with frozen-thawed spermatozoa

From data shown in Table 3, it can be observed that both boars showed greater penetration rates when oviductal cells were present during IVF ($P < 0.01$), and a tendency ($P = 0.052$) was observed for the mean number of spermatozoa. In this experiment neither boar

Table 2
IVF results for immature oocytes fertilized with different sperm concentrations (cells/ml) without (−) or with (+) POEC[a,b]

Cells/ml	POEC	*n*	%PEN	S/O	%MON[c]
5×10^5	−	145	17.24 ± 3.15 a	1.24 ± 0.11 a	84.00 ± 7.48 a
	+	149	37.58 ± 3.98 bc	1.51 ± 0.10 a	65.50 ± 6.53 a
1×10^6	−	161	31.06 ± 3.66 ac	1.786 ± 0.14 ab	54.00 ± 7.12 ab
	+	160	48.13 ± 3.96 b	2.90 ± 0.30 c	36.36 ± 5.52 b
5×10^6	−	134	56.00 ± 4.30 b	2.66 ± 0.20 bc	32.00 ± 5.42 b
	+	159	81.76 ± 3.07 d	3.87 ± 0.21 d	11.54 ± 2.81 c
Source of variability					
Cells/ml			<0.001	<0.001	<0.001
POEC			<0.001	<0.001	<0.001
Cells/ml by POEC			0.492	0.242	0.944

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O) and monospermy rate (%MON).
[b] Different letters in the same column indicate significantly different values ($P < 0.05$).
[c] From the penetrated oocytes.

nor POEC had any influence on male pronuclear formation. However, it was detected an individual effect of the boars ($P < 0.01$) on monospermy rate, although this parameter was similar between POEC+ and POEC− groups.

3.4. Effect of pre-culture spermatozoa in oviductal cell monolayers on in vitro fertilization results

For the results corresponding to the low penetrability boar (Table 4), the treatment given to the spermatozoa had an effect on all the studied parameters ($P < 0.05$). As in previous

Table 3
Effect of boar and POEC on the IVF results with frozen-thawed semen[a,b]

Boar	POEC	*n*	%PEN	S/O	%MPN[c]	%MON[c]
A	−	129	15.50 ± 3.2 a	1.21 ± 0.12 ab	52.63 ± 11.77	84.21 ± 8.59 a
	+	118	45.76 ± 4.61 b	1.32 ± 0.08 a	49.06 ± 6.93	75.47 ± 5.97 a
B	−	120	69.17 ± 4.23 c	2.04 ± 0.13 b	59.26 ± 5.49	40.74 ± 5.49 b
	+	108	87.04 ± 3.25 d	2.80 ± 0.21 c	53.19 ± 5.17	34.04 ± 4.91 b
Source of variability						
Boar			<0.001	<0.001	0.485	<0.001
POEC			<0.001	0.052	0.531	0.283
Boar by POEC			0.111	0.147	0.871	0.887

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), rate of male pronuclear formation (%MPN) and monospermy rate (%MON).
[b] Different letters in the same column indicate significantly different values ($P < 0.05$).
[c] From the penetrated oocytes.

Table 4
IVF results for the low penetrability boar employing porcine spermatozoa pre-cultured different times in presence of oviductal cells monolayers[a,b]

Sperm pre-incubation	Fertilization in oviductal cells	*n*	%PEN	S/O	%MPN[c]	%MON[c]
2 h POEC	+	97	62.89 ± 4.93 a	4.11 ± 0.44 a	83.61 ± 4.78 a	26.23 ± 5.68 a
0 h POEC	+	94	60.64 ± 5.07 a	2.01 ± 0.19 b	63.16 ± 6.45 b	52.63 ± 6.67 b
Control	−	108	41.67 ± 4.77 b	3.22 ± 0.33 ab	82.22 ± 5.76 ab	24.44 ± 6.48 a
Source of variability						
Treatment			0.003	<0.001	0.017	0.002

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), rate of male pronuclear formation (%MPN) and monospermy rate (%MON).
[b] Different letters in the same column indicate values significantly different ($P < 0.05$).
[c] From the penetrated oocytes.

experiments, the penetration rate was higher in those oocytes fertilized in presence of POEC than in those without oviductal cells. Moreover, the sperm pre-incubation in POEC before IVF had a positive effect on the number of spermatozoa per oocyte; that was twice the quantity in 2 h POEC group than in 0 h POEC group. So sperm pre-incubation had a negative effect on monospermy that was higher in oocytes fertilized in presence of POEC and with no pre-cultured spermatozoa. In vitro fertilization with spermatozoa pre-cultured 2 h in IVF medium without POEC reached a penetration rate of 5.56 ± 3.15 and 1 spermatozoa per penetrated oocyte ($n = 54$, data not shown in table).

With regard to the high penetrability boar (Table 5) sperm pre-incubation before IVF did not affect either the rate of penetration or the monospermy rate. The S/O was higher ($P < 0.01$) in the oocytes fertilized with spermatozoa pre-cultured 2 h in oviductal cells but no differences appeared between those pre-incubated 0 h and the control group. An effect of the treatment was observed on the male pronuclear formation rate ($P < 0.01$). In this case the penetration rate with spermatozoa pre-cultured 2 h in IVF medium without POEC was again extremely low, 9.09 ± 3.30 with 2.00 ± 0.43 spermatozoa per penetrated oocyte ($n = 77$, data not shown in table).

Table 5
IVF results for the high penetrability boar employing porcine spermatozoa pre-cultured different times in presence of oviductal cells monolayers[a,b]

Sperm pre-incubation	Fertilization in oviductal cells	*n*	%PEN	S/O	%MPN[c]	%MON[c]
2 h POEC	+	98	97.96 ± 1.44	13.13 ± 1.32 a	66.67 ± 4.84 a	10.42 ± 3.13
0 h POEC	+	93	96.77 ± 1.84	8.62 ± 0.97 b	88.89 ± 3.33 b	14.44 ± 3.73
Control	−	108	99.07 ± 0.93	6.54 ± 0.39 b	82.24 ± 3.71 ab	5.61 ± 2.23
Source of variability						
Treatment			0.513	<0.001	<0.001	0.115

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), rate of male pronuclear formation (%MPN) and monospermy rate (%MON).
[b] Different letters in the same column indicate significantly different values ($P < 0.05$).
[c] From the penetrated oocytes.

Table 6
Results with in vitro matured oocytes pre-cultured 4, 2 or 0 h with oviductal cells before IVF[a,b]

Oocyte pre-incubation	Fertilization in oviductal cells	*n*	%PEN	S/O	%MPN[c]	%MON[c]
4 h POEC	+	113	48.67 ± 4.72	1.40 ± 0.10 a	89.09 ± 4.24 a	74.55 ± 5.93 a
2 h POEC	+	98	50.00 ± 5.08	1.87 ± 0.20 ab	71.43 ± 6.52 b	55.10 ± 7.18 ab
0 h POEC	+	113	61.95 ± 4.59	1.94 ± 0.14 b	88.57 ± 3.83 a	47.14 ± 6.01 b
Control	−	112	58.93 ± 4.67	2.39 ± 0.24 b	77.27 ± 5.20 ab	48.48 ± 6.20 b
Source of variability						
Treatment			0.126	<0.001	0.034	0.009

[a] Number of oocytes (*n*), rate of penetration (%PEN), mean number of spermatozoa per penetrated oocyte (S/O), rate of male pronuclear formation (%MPN) and monospermy rate (%MON).
[b] Different letters in the same column indicate significantly different values ($P < 0.05$).
[c] From the penetrated oocytes.

3.5. *Effect of POEC-oocyte pre-incubation on IVF results*

The penetration rates were similar in all groups but an effect of the treatment was observed for the rest of the parameters studied ($P < 0.05$; Table 6). The oocytes pre-cultured 4 h in POEC before IVF showed the best IVF results with the lowest number of spermatozoa per penetrated oocyte and the highest monospermy rate. The rest of the groups showed similar results for both S/O and %MON.

4. Discussion

The oviductal reservoir serves a number of functions including prevention of polyspermic fertilization which is achieved both by the role of isthmus in controlling number of spermatozoa that reach the oocyte (Suarez, 1998) and oviductal fluid secreted by the cells. In that microenvironment, oocyte and sperm find physiological conditions, and in vivo fertilization of pig oocytes is monospermic in over 95% of instances (Hunter, 1972). In this way, oviductal cell cultures have been employed in different species in order to imitate this environment, since the epithelial cells maintain their secretory activity in culture as indicated by electron microscopy and immunocytochemistry (Park and Sirard, 1996). Moreover, porcine oviductal cells remain morphologically and biochemically highly active in culture (Nagai and Moor, 1990). From these observations we suppose that the in vitro fertilization medium is modified by the same in vivo secreting products from the oviductal cells. These oviductal secretions could modify both gametes independently or the IVF process itself.

Obviously in porcine IVF, where polyspermy has been a classic problem (Coy et al., 1993; Nagai, 1996; Rath, 1992), the possible effect on monospermy rates has driven many investigators to include oviductal explants in their IVF systems as another tool for improving the results and output of the process (Dubuc and Sirard, 1995; Gadea et al., 1998; Nagai and Moor, 1990). From the current papers in this field, although there are differences between them, and from this one, it can be transpired that the oviductal cells improve monospermy rate. This enhancement could be achieved operating on the oocyte, on the sperm or on

the whole IVF system, for instance allowing a reduction in the number of spermatozoa confronting the oocytes at fertilization because of their effect on penetration rate. From our results, the latter point seems to be certain, since presence of oviductal cells increased oocyte penetration rate and number of spermatozoa per oocyte regardless of the oocyte maturational stage (mature or immature, experiments 1 and 2) and the sperm nature (fresh in experiments 1 and 2, or frozen as in experiment 3). This observation has also been described in bovine (Martus et al., 1998) where acceptable fertilization rates with low sperm concentration were achieved including a partially purified oviductal glycoprotein in the IVF protocol. This improvement in the results could be employed to achieve better fertilization when we have to work with medium quality sperm, caused by previous treatments such as the frozen-thawing process (experiment 3) or in medium-low fertility boars (experiment 4, Table 4). It is known that frozen-thawed spermatozoa are more sensitive to in vitro culture than fresh spermatozoa, thus sperm motility decreases more rapidly in vitro (Lefebvre and Suarez, 1996). So the introduction of oviductal cells in the IVF system would be useful to overcome this disadvantage. Moreover, the oviductal cells from prepubertal gilts, as in the present work, seem to be sufficiently differentiated to exert capacitation-inducing effects on boar spermatozoa and have a high potential to resume growth in in vitro culture (Fazeli et al., 1999).

On the other hand we infer from experiments 1–3 that as penetration rate and number of spermatozoa increase, due to the employment of POEC, monospermy rate is lower or at best similar to those groups where POEC is absent, but in any case the monospermy rate from POEC+ groups was higher than the POEC− groups. So, is that wrong that porcine oviductal cells increase the monospermy rate, as has been previously suggested (Dubuc and Sirard, 1995; Nagai and Moor, 1990)? One possible explanation to this controversy could be the longer coincubation time (18 h) between oocyte and spermatozoa in experiments 1–3 (Coy et al., 1993). Results from Kano et al. (1994) show that the rate of monospermic oocytes co-cultured with epithelial cells increases between 4 and 8 h after insemination and decreases when this period of time increases. Another reason could be that gametes need a previous contact with oviductal cells to permit cells to act on them for enough time before contact during IVF. These hypotheses led us to change the coincubation time, to 6 h instead of 18 h, and pre-incubate both gametes separately (spermatozoa in experiment 4 and oocytes in experiment 5).

Under these experimental conditions, we again observed in experiment 4 that presence of POEC during the IVF process increased oocyte penetrability, so that we come to the conclusion that the beneficial effect of POEC consisted only of the possibility of using low sperm concentrations or low quality semen for IVF. However, if we analyze data conscientiously, then we observe that sperm pre-incubated 2 h with POEC showed a similar penetration rate to those pre-incubated 0 h but the former showed a higher number of spermatozoa per oocyte and lower monospermy rate (Table 4). It has been described that during the period of contact with oviductal cells sperm viability is maintained and capacitation proceeds at a slower rate, so the fertilizable life increases (Smith, 1998). In our experiment, it means that a higher number of released sperm reached the oocytes increasing thus the number of spermatozoa per penetrated oocyte. In fact, we observed highly motile spermatozoa after many hours of culture as other researchers have described in several species (Demott and Suarez, 1992; Park and Sirard, 1996; Pollard et al., 1991; Suarez et al., 1991). Besides, the penetrability results for spermatozoa pre-cultured 2 h in IVF medium without POEC were

certainly low, possibly due to a premature spermatozoa death (Matás, 1996). On the other hand, when we compare monospermy results from 0 h POEC with the control group, we detect a significant beneficial effect of POEC. Therefore, it seems that the effect of oviductal cells depends on the coincubation time of the gametes, so we suggest that POEC allow us to work with lower number of sperm and increase monospermy rate when coincubation time is limited to 6 h. These results would be similar to that from other research groups (Dubuc and Sirard, 1995; Nagai and Moor, 1990).

The last experiment results show that under the same IVF conditions (boar, sperm concentration, time of culture, etc.) the oocytes pre-incubated 4 h showed higher monospermic penetrations than the other groups. This finally drives us to a clear beneficial effect of oviductal cells on the final IVF output showing that oviductal cells (secretions and cells) have, in some way, a preventative action on oocytes against an excessive sperm penetration. With reference to this oocyte protection it has been suggested that oviductal secretion promotes a hardening of the zona pellucida and is required for the complete cortical granule release (Kim et al., 1996), although recently Wang et al. (1998) have indicated that in vitro matured and in vivo matured pig oocytes possess equal ability to release cortical granule. Starting from the basis that porcine oviductal secretory proteins associate with the zona pellucida and other parts of the oocyte (Buhi et al., 1993), our thinking is that the two prevailing views regarding oviductal function (passive and active) (Gandolfi, 1995) are complementary. In this way, oviductal cell monolayers would improve the monospermy rate both providing an optimal environment and by secretion of a variety of molecules which sustain and regulate or enhance events preceding and during fertilization. Martus et al. (1998) have recently found a beneficial effect of purified oviductal glycoprotein on the bovine oocytes pre-incubating them before IVF, but not on sperm and further studies would be necessary in this field.

5. Conclusion

It is possible to improve the monospermic penetration in pigs by a 4 h oocyte pre-culture in POEC and only 6 h of contact between gametes. Moreover, porcine oviductal cells let us work with low sperm concentrations or even with low quality sperm, increase oocyte penetrability by favoring and improving the sperm viability and have a beneficial effect on oocytes, improving the final rate of monospermy. From the results of this study we can conclude that the fertilization parameters and monospermy depend on factors such as the sperm concentration, individual differences between boars, coincubation time and period of pre-culture in oviductal cells before IVF.

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