ELSEVIER

EFFECTS OF MATURATIONAL STAGE, CUMULUS CELLS AND COINCUBATION OF MATURE AND IMMATURE CUMULUS-OOCYTE COMPLEXES ON IN VITRO PENETRABILITY OF PORCINE OOCYTES

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ABSTRACT

The in vitro penetrability of porcine oocytes is conditioned by several factors, some of which remain unclear. Knowledge of the different effects of the cellular components involved in penetrability would no doubt serve to simplify laboratory IVF methods. This study was designed to evaluate the effects of the following factors on penetrability: oocyte maturational stage, the presence of isolated or oocyte-attached cumulus cells, and coincubation of in vitro-matured and immature oocytes.

Immature oocytes and oocytes matured in Waymouth medium were obtained from non atretic follicles and fertilized in TCM 199 medium. Sperm-rich fractions were collected by the gloved hand method and semen was used for IVF at a final concentration of 1 x 10^6 cells/mL in all experiments. Under the same conditions of IVF, the penetrability of the immature cumulus-oocyte complexes (COCs) was significantly lower than that of mature COCs, in terms of penetration rate and mean number of sperm per penetrated oocyte. This difference was abolished when the oocytes were denuded, leading to similar penetration rates. Coincubation of mature and immature COCs reduced the penetrability of immature COCs compared with that observed when these were incubated in isolation. However, neither the addition of isolated cumulus cells from decumulated mature oocytes nor the addition of denuded mature oocytes to immature COCs modified the penetration rate.

These findings suggest that the presence of surrounding cumulus cells is mainly responsible for the differences observed in penetrability, regardless of the maturational stage of the oocyte. Moreover, when mature and immature COCs are coincubated, penetrability of immature COCs is diminished by the effects of the mature COC and not by the independent actions of the cellular components.

Key words: IVM, IVF, penetrability, cumulus cells, porcine

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INTRODUCTION

Recent efforts to standardize porcine in vitro maturation/fertilization (IVM/IVF) systems include the use of chemically defined, serum-free maturation media such as NCSU (13, 14). However, several other factors involved in the fertilization process (9, 10, 22) have not yet been identified, let alone standardized. This hinders the comparison of results among research teams and dictates the need to perform large numbers of experiments with the corresponding large number of replicates before embarking on any type of research in this field. Boar selection (35, 36) and appropriate sperm concentration (6, 8, 37) are both frequent problems encountered. Of considerable interest would be the use of immature oocytes to reduce the time used in selecting the inseminate, based on the suggestion that immature porcine oocytes are fully capable of interacting with sperm and undergo penetration (15). Further, it has been established that the penetrability of immature oocytes is similar to that of mature oocytes (19) despite the fact that, given the insufficient maturity of the cytoplasm, the immature oocyte is not able to decondense the sperm head (25). It should be noted, however, that even studies comparing the penetrability of mature and immature oocytes under the same precise experimental conditions yield controversial results.

In other in vitro experiments, the use of oocytes with or without cumulus cells may be a determining factor when comparing penetration. Mattioli et al. (24) showed the importance of intracellular cooperation between cumulus cells and oocytes during in vitro maturation for oocyte penetrability. Galeati et al. (15) also reported that the presence of cumulus cells during maturation is fundamental for pig oocyte penetrability. Nonetheless, data on the effects of cumulus cells on the in vitro fertilization process are scarce (9, 16, 17). The time-consuming task of decumulation of oocytes to achieve maximum penetrability has yet to be proven necessary.

On occasion, the use of oocytes matured in vitro gives rise to the simultaneous presence of mature and immature oocytes in the same culture dish. In vitro maturation systems generally show a narrow range of Metaphase II rates (78 to 98%; 2, 3, 16, 18, 33) indicating that there is always a variable number of immature COCs or oocytes during IVF. To date, the possible influence of this coincubation of immature and mature oocytes on in vitro penetrability remains unknown. Similarly, the effects of cumulus cells isolated from mature oocytes or decumulated mature oocytes on the in vitro penetrability of immature COCs have not yet been explored.

The aim of the present study was to examine the effects of the following factors on IVF in pigs: oocyte maturational stage, the presence or absence of isolated or oocyte-attached cumulus cells, and the coincubation of in vitro-matured and immature oocytes.

MATERIALS AND METHODS

Media

Unless otherwise stated, all the chemicals used in this study were purchased from Sigma-Aldrich Química (Madrid, Spain). The medium used for oocyte maturation was Waymouth MB 725/1 supplemented as previously described (11, 38) with 10 IU/mL eCG (Foligon, Intervet International B.V., Boxmeer, Holland), 10 IU/mL hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), 1 μg/mL estradiol-17β, 10% (v/v) fetal calf serum (Biological Industries, Beitz Haemek, Israel) and 10% (v/v) porcine follicular fluid.

The fertilization medium was TCM 199 (7) supplemented with 0.91 mM Na-pyruvate, 2.92 mM Ca-lactate, 3.05 mM glucose, 50 IU/mL penicillin G, 30 μg/mL streptomycin sulphate and 12% (v/v) fetal calf serum.

Collection and Culture of Oocytes

Ovaries were isolated from prepubertal gilts just after slaughter at a local abattoir and transported to the laboratory in saline (0.9% wt/v NaCl) containing 100 mg/L kanamycin and 50 mg/L polymyxin B sulfate at 37°C. Specimens were washed once in 0.04% cetrimide solution and twice in saline. Cumulus-oocyte complexes, obtained from nonatretic follicles (3 to 6 mm diameter), were rinsed twice in Dulbecco's phosphate buffered saline supplemented with 4 mg/mL polyvinyl alcohol (PBSDm, Sigma) in 35 mm plastic Petri dishes. Oocytes for in vitro maturation were twice washed again in maturation medium, previously conditioned for a minimum of 3 hours in a 5% CO_2 air atmosphere. Oocytes for in vitro fertilization as immature oocytes were rinsed in fertilization medium instead of maturation medium.

In vitro maturation was performed by culturing clumps of 20 oocytes in 100 μL droplets of maturation medium for 20 to 22 h at 38.5°C in a 5% CO_2 air atmosphere. The oocytes then were transferred to fresh maturation medium with no hormonal supplements, washed twice and cultured for a further 20 to 22 h (1).

Isolation of Cumulus Cells

After in vitro maturation, batches of COCs cultured for 44 hours were repeatedly pipetted in PBSDm to strip the associated cumulus cells. Oocytes were removed and the wash containing the cumulus cells was centrifuged at 440 x g for 10 min. The resultant pellet was resuspended in TCM 199 and the concentration adjusted using a hemocytometer to a final concentration of 30.000 cells/Petri dish.

In Vitro Fertilization

Sperm-rich fractions were collected by the gloved hand method. Spermatozoa were diluted to 3 x 10^7 cells/mL in Beltsville thawing solution extender (BTS) and stored at 15°C for 24 hours. The sperm samples then were washed by centrifugation

(50 x g, 3 min) to remove heavy particles. The supernatant was concentrated (1250 x g, 4 min) and the pellet resuspended in TCM 199 to the desired final concentration (1 x 10^6 cells/mL). Spermatozoa in a final volume of 100 µL were added to Petri dishes containing 2 mL of fertilization medium and 20 oocytes.

After 18 h the oocytes were fixed, stained with 1% (wt/v) lacmoid and examined at a magnification x 400 for evidence of sperm penetration using phase contrast microscopy.

Experimental Design and Statistical Analysis

Experiment 1

This experiment was designed to study the in vitro penetrability of mature, immature and coincubated mature and immature oocytes. Groups of 20 in vitro-matured COCs (usually one group, 20 oocytes per replicate and boar), 20 immature COCs (usually one group, 20 oocytes per replicate and boar) and 10 mature/10 immature COCs (usually two groups, 40 oocytes per replicate and boar) were rinsed in fertilization medium and were fertilized in vitro. Insemination was achieved using ejaculates from three different boars (A, B, C) at a concentration of 1 x 10^6 cells/mL. In total, 1923 COCs were inseminated in 8 replicate experiments.

Experiment 2

The second experiment was designed to determine whether the different penetrability rates observed in Experiment 1 were attributable to the presence of cumulus cells from mature and immature oocytes or were alternatively dependent on maturational stage. At the end of the maturation period, 50% of the oocytes were denuded by pipetting and placed in Petri dishes for IVF, while the remaining 50% were inseminated without previous manipulation (Figure 1 a, b). On the same day and in the same manner, 50% of the immature oocytes obtained were denuded before IVF (Figure 1 c, d). All the oocytes were inseminated with semen from the same boar at a concentration of 1x10^6 cells/mL. This experiment was performed in quadruplet using 607 oocytes.

Experiment 3

This experiment was designed to determine whether the reduced penetration rate was attributable to the presence of mature oocytes in the culture dish or to that of the cumulus cells associated with mature oocytes. Denuded mature oocytes were coincubated with immature COCs, and cumulus cells from mature oocytes were incubated with immature COCs at a concentration of 1500 cells/oocyte. This concentration was decided by denuding mature oocytes and dividing the number of cells obtained by the number of oocytes denuded. Immature COCs incubated alone served as controls.

Semen from one boar was used as the inseminate at a concentration of $1x10^6$ cells/mL. This experiment was performed four times using 748 oocytes.

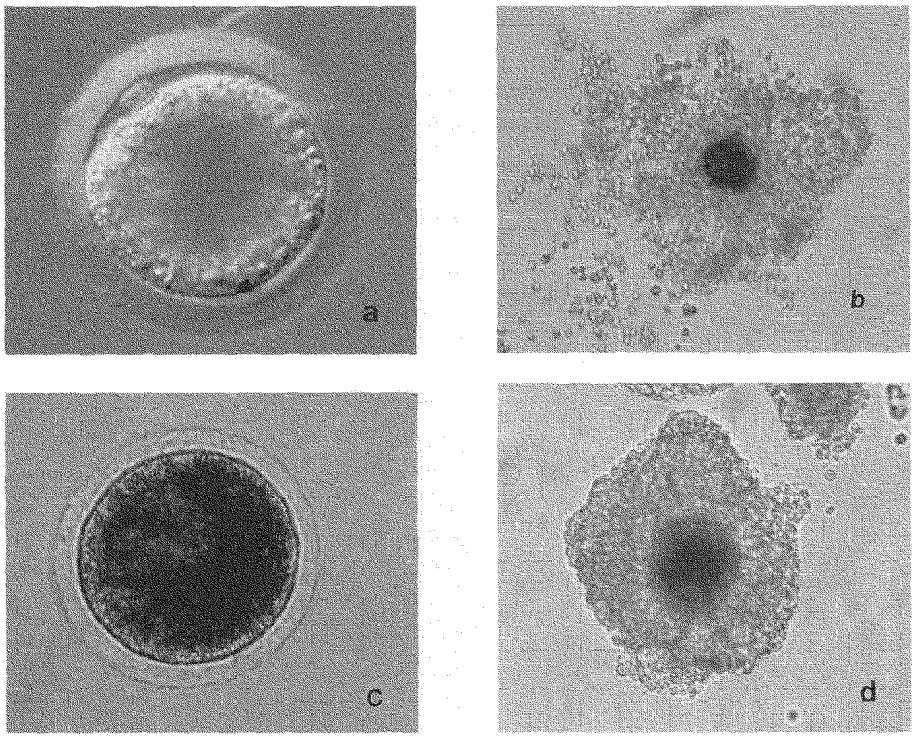

Figure 1. a) Denuded mature oocyte (x 400), b) Mature COC (x 100), c) Immature denuded oocyte (x 400), d) Immature COC (x 200). a) Hoffman system; b), c) and d) phase contrast.

Statistical Analysis

Penetration rates were expressed as percentages and modeled according to the binomial variable method. The number of sperm per penetrated oocyte was expressed as the mean ± SEM. Data provided by Experiments 1 and 3 were analyzed by one-way ANOVA using the General Linear Model's Systat program. Data derived from Experiment 2 were subjected to two-way ANOVA, considering maturational stage and the presence of cumulus cells as the main effects. The Tukey test was used to compare data when a significant effect was detected. The level of significance was set at $P < 0.05$.

RESULTS

Experiment 1

The penetrability of immature oocytes was significantly lower than that of the mature oocytes for each boar used. Moreover, the penetration rate of immature oocytes in the coincubation group was less than that recorded for the immature oocytes inseminated in isolation. These differences were significantly different ($P < 0.05$) with the exception of inseminations performed using semen from boar C (Table 1).

Table 1. Number of penetrated oocytes, penetration rate (PEN), mean number of sperm per penetrated oocyte (S/O) according to the boar used to obtain the semen.

Boar		Immature COCs	Mature COCs	Coincubated	
				Immature COCs	Mature COCs
A	N	142	147	146	164
	PEN	53.52 ± 4.20^{a}	97.28 ± 1.25^{b}	39.73 ± 4.06^{c}	93.29 ± 1.96^{b}
	S/O	3.48 ± 0.41^{w}	8.35 ± 0.56^{x}	2.94 ± 0.33^{y}	12.72 ± 0.74^{z}
B	N	197	158	175	163
	PEN	69.54 ± 3.29^{a}	96.84 ± 1.40^{b}	50.86 ± 3.88^{c}	98.16 ± 1.06^{b}
	S/O	4.72 ± 0.48^{x}	12.54 ± 0.59^{y}	2.98 ± 0.32^{x}	11.19 ± 0.70^{y}
C	N	200	133	157	141
	PEN	29.50 ± 3.67^{a}	48.87 ± 4.35^{b}	18.47 ± 3.11^{a}	56.74 ± 4.19^{b}
	S/O	2.23 ± 0.30^{wx}	2.52 ± 0.27^{x}	1.34 ± 0.13^{w}	2.47 ± 0.20^{x}

[a, b, c, w, x, y, z] Superscripts in the same row indicate significantly different values ($P < 0.05$).

The penetration rate of mature oocytes and the number of sperm per penetrated oocyte were the same in the isolation and coincubation groups with the exception of oocytes fertilized using sperm from Boar A. In this last case, the number of sperm per penetrated oocyte increased (8.35 vs 12.72).

Experiment 2

The penetration rate of immature oocytes obtained from immature COCs was significantly lower than that corresponding to the denuded immature oocytes (Table 2). The latter achieved penetration rates close to 100% with respect to values obtained using mature oocytes. The presence of cumulus cells had no effect on the penetration of mature oocytes although the mean number of sperm per oocyte increased.

Table 2. Number of penetrated oocytes, penetration rate (PEN) and mean number of sperm per penetrated oocyte (S/O) corresponding to intact COCs and denuded oocytes.

	Immature		Mature	
	With cumulus	Denuded	With cumulus	Denuded
N	157	131	206	113
PEN	59.24 ± 3.93^{a}	96.95 ± 1.51^{b}	100^{b}	97.35 ± 1.52^{b}
S/O	2.74 ± 0.24^{x}	5.22 ± 0.29^{x}	22.25 ± 0.76^{y}	5.13 ± 0.42^{x}

$^{a, b, x, y}$ Superscripts in the same row indicate significantly different values ($P < 0.05$).

Experiment 3

No differences in penetration rate and mean number of sperm per penetrated oocyte were shown by the different immature oocyte groups, irrespective of the presence of denuded mature oocytes or mature cumulus cells (Table 3).

Table 3. Number of penetrated oocytes, penetration rate (PEN) and mean number of spermatozoa per oocyte (S/O) corresponding to immature COCs incubated in the presence of denuded mature oocytes or cumulus cells isolated from mature oocytes.

	Immature			Mature	
	I	I_{MD+I}	I_{CM}	MD	MD_{MD+I}
N	154	136	175	114	169
PEN	51.95 ± 4.04^{a}	41.18 ± 4.24^{a}	45.71 ± 3.78^{a}	96.49 ± 1.73^{b}	98.82 ± 0.83^{b}
S/O	1.98 ± 0.14^{x}	2.28 ± 0.25^{x}	2.20 ± 0.19^{x}	5.30 ± 0.42^{y}	5.00 ± 0.24^{y}

I: Immature COCs.
I_{MD+I}: Immature COCs from the immature COC+denuded mature oocyte culture.
I_{CM}: Immature COCs + cumulus cells from mature oocytes.
MD: Mature denuded oocytes.
MD_{MD+I}: Mature denuded oocytes from the immature COC+denuded mature oocyte culture.
$^{a, b, x, y}$ Different superscripts in the same row indicate values significantly different ($P < 0.01$).

DISCUSSION

The results of the first experiment indicate clear differences in the penetrability of oocytes matured in vitro and immature oocytes fertilized under the present conditions; and lower penetration rates of immature oocytes coincubated with mature COCs with respect to the penetration rates of oocytes fertilized in isolation. These findings could be attributed to the maturational stage of the oocyte (Metaphase II or germinal vesicle phase), or to the presence of cumulus cells that are expanded or attached to the oocyte in each phase respectively.

The influence of maturational stage of the oocyte on penetrability was investigated by several authors (15, 19, 25, 29, 34). However, results are conflicting and inconclusive due to the different methodologies and experimental designs used. The most similar methodology to ours was that used by Matás et al. (20), who compared the penetrability of ovulated and immature oocytes. These authors reported no difference between these types of gametes, probably because of the high sperm concentration used. In contrast, the present findings suggest a difference in the penetrability of immature oocytes and those matured in vitro when COCs are used.

Cumulus cells from mature COCs may play a role in penetrability (Experiment 1, Table 1) since immature COCs were less penetrated than those fertilized when incubated alone. But there could be other explanations related to the effect of the mature oocytes or the whole mature COC on the in vitro penetrability of such immature co-incubated oocytes. The results of the second experiment showed that cumulus cells and not the nuclear maturational stage of the oocyte were responsible for the difference in penetrability between mature and immature oocytes observed in Experiment 1. Saeki et al. (30) showed that the cumulus cells of mature oocytes exerted a greater effect on penetrability than did cumulus cells of immature oocytes in the cow. Similar effects might occur in pigs, since several authors report that certain proteins found in the expanded cumulus cells of mature oocytes are able to induce or promote the boar sperm acrosome reaction (23), possibly favoring sperm penetration. Further, expanded cells of mature oocytes are known to enhance sperm motility and to preserve oocyte quality (34). We propose that cumulus cells from immature oocytes are unable to exert this effect and consequently give rise to diminished penetrability, as occurs in the IVF of human oocytes (4).

Denuding the oocytes led to similar penetration rates in both mature and immature gametes (Table 2). Hence, denuded immature oocytes showed higher penetrability values than did immature COCs. In our opinion, cumulus cells attached to the immature oocyte could form a barrier to sperm penetration although similar penetration rates were reported for denuded and non-denuded immature oocytes (21). Again, the different experimental conditions (sperm concentration mainly) might explain this discrepancy.

Some authors report no changes in penetrability after denuding oocytes matured in vitro (32). In contrast, others showed that denuded mature oocytes are less penetrable by frozen-thawed spermatozoa than those with an intact cumulus at the time of IVF (34). The manipulation of oocytes before denuding causes premature exocytosis of cortical granules (15) and this could explain the diminished penetrability of the denuded mature oocyte. Moreover, as already indicated, the cumulus cells of mature oocytes may enhance sperm motility or promote the acrosome reaction favoring penetration (34). The findings of Zheng and Sirard (39), Kikuchi et al. (17) and of our second experiment (Table 2) indicate that denuding mature oocytes leads to reduced penetrability in terms of the number of sperm per penetrated oocyte. These results contradict a previous report from our laboratory (9) in which we compared the same variable (number of spermatozoa per oocyte) using oocytes matured in vivo with an intact cumulus and denuded in vivo matured oocytes. As

suggested in the paper, ovulated oocytes, which come into contact with oviductal glycoproteins before they are harvested, could in some way be protected against polyspermy, compared to oocytes matured in vitro.

Confirmation of the present findings should establish whether mature or immature, or non-denuded or denuded oocytes are most suitable for use in IVF experiments. However, the results of Experiment 1 give rise to the question: Why were the immature oocytes coincubated with mature oocytes less penetrated than the immature oocytes incubated alone? One possible explanation, could be that the mature oocyte, or cumulus-oocyte complex, exerts some kind of inhibitory effect on the penetrability of immature oocytes. In Experiment 3, where immature COCs were coincubated with denuded mature oocytes no difference in penetrability was found with respect to that recorded for the immature COCs incubated alone. Similarly, there was no difference between the penetrability of immature oocytes incubated with cumulus cells from the same number of mature oocytes, and control oocytes incubated alone. This would suggests that the entire mature cumulus-oocyte complex, including the extracellular matrix, and not its isolated cellular components (cumulus cells, oocyte) hinders the penetration of the coincubated immature oocytes, favoring sperm penetration of the mature oocytes. Indeed, there is abundant evidence of significant metabolic co-operation between oocytes and follicular cells throughout the maturation process (12, 24, 25, 26, 27, 28, 29). This relationship could persist at least until the initial stages of IVF. Such interaction not only provides nutritional benefits for the oocyte, but may also condition other physiological processes such as sperm penetration. It has been proposed that the cumulus-oocyte complex enhances oocyte penetrability by secreting substances that promote penetration (30) and the acrosome reaction (4, 23, 31), favoring sperm capacitation (34), or by simply sequestering sperm as reported for rat oocytes (5). This may explain why immature oocyte penetrability was reduced in the coincubation group in Experiment 1.

In conclusion, our findings suggest that the presence of cumulus cells and not the maturational stage is mainly responsible for the differences observed in the in vitro penetrability of mature and immature porcine oocytes; the coincubation of immature and mature COCs reduces the in vitro penetrability of the immature oocytes only and; this effect is mediated by the mature COC and not independently by the cumulus cells or the oocyte.

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