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Environment and medium volume influence *in vitro* fertilisation of pig oocytes

P. Coy[1], *E. Martínez*[2], *S. Ruiz*[1], *J.M. Vázquez*[2], *J. Roca*[2] *and J. Gadea*[2]
University of Murcia, Murcia, Spain

Summary

Oviductal oocytes ($n = 795$) were obtained from ovulation-induced prepubertal gilts. In the first experiment, different parameters related to *in vitro* fertilisation (IVF) were compared in the presence and absence of cumulus matrix (which is shed with the oocytes at ovulation.) The results show that the presence of this matrix is beneficial because the rates of fertilisation (69%) and monospermy (number of monospermic oocytes/total number of healthy mature oocytes; 42%), and the median number of spermatozoa per oocyte (1.52 ± 0.06), were improved with respect to those obtained in its absence (54%, 22% and 2.33 ± 0.08, respectively; $p < 0.01$). In the second experiment the effect of two different volumes of co-incubation medium (2 ml and 0.4 ml) on the same parameters of porcine IVF were compared. No significant differences between volumes were observed, except in the mean number of spermatozoa per oocyte and the percentage of dispermic oocytes.

Keywords: Cumulus, *In vitro* fertilisation, Medium volume, Porcine

Introduction

Successful *in vitro* fertilisation of pig eggs (offspring born) was first reported by Cheng *et al.* (1986). Subsequently, numerous workers have tried to improve the efficiency of *in vitro* fertilisation in the pig (Nagai *et al.*, 1988; Mattioli *et al.*, 1989; Nagai & Moor, 1990; Park & Pursel, 1991). Although fertilisation rates are extremely high (80–100%), the probability of obtaining viable embryos is very low due to the high rate of polyspermic fertilisation.

In previous work, we studied the effect of co-incubation time (Coy *et al.*, 1993*a*) and sperm concentration (Coy *et al.*, 1993*b*), observing that both factors have a significant influence on the results of pig IVF but are not the only factors.

The environment during IVF in pigs is quite different from that of the oviduct (Mattioli *et al.*, 1989). For example, *in vivo*, cumulus cells and intercellular matrix are present at least at the beginning of the oocyte's stay in the oviduct. The effect of the presence of cumulus cells on the results of IVF varies among species. In the rabbit and hamster cumulus cell removal had no effect on the proportion of eggs fertilised *in vitro* (Chang *et al.*, 1971; Fraser *et al.*, 1971; Miyamoto & Chang, 1972). Removal of cumulus cells reduced the fertilisation rate of mouse eggs (Cross & Brinster, 1970; Miyamoto & Chang, 1972). Granulosa cells obtained from the follicular wall increased the fertilisation rate and decreased polyspermy of bovine oocytes (Fukui & Ono, 1989). Cumulus cells influenced the maturation and penetrability of these oocytes also (Shioya *et al.*, 1988). In pigs, cumulus cells may improve the rates of fertilisation, but no data are available about the effects on monospermy (Kikuchi *et al.*, 1991).

Another factor that may influence the results of IVF is the volume of co-incubation medium, with the confounding effects of sperm number and sperm concentration (Mattioli *et al.*, 1989). Medium volume affected the rates of fertilisation and monospermy in rats (Niwa & Chang, 1974).

The present studies were designed to evaluate the

All correspondence to: P. Coy, Departamento de Biología Animal (Fisiología Animal), Facultad de Veterinaria, Campus de Espinardo, Universidad de Murcia, Aptdo. 4021, Murcia E-30071, Spain. Fax: 34-68 305101.
[1] Department of Animal Biology, Veterinary Faculty, University of Murcia, Aptdo. 4021, Murcia E-30071, Spain.
[2] Department of Animal Pathology, Veterinary Faculty, University of Murcia, Aptdo. 4021, Murcia E-30071, Spain.

effects of cumulus cells and intercellular matrix (after exposure to the oviductal environment) and of medium volume on the efficiency of pig IVF.

Materials and methods

Culture media

Dulbecco's phosphate-buffered saline (PBSDm) supplemented with 4 mg/ml bovine serum albumin (BSA, fraction V; Sigma, St Louis, Mo.), 0.36 mM pyruvate (Merck, Darmstadt, Germany), 5.5 mM glucose (Merck) and 70 μg/ml kanamycin (Sigma) (Mattioli *et al.*, 1988), pH 7.4, was used for flushing oviducts and manipulating oocytes. Sperm washing medium (SWM) was saline (0.9% NaCl) containing 1 mg/ml BSA, pH 7.2 (Yanagimachi, 1981). Sperm capacitation medium (CM) was TCM 199 (Earle salts, Boehringer, Mannheim, Germany) supplemented with 12% heat-inactivated fetal calf serum (Gibco, Grand Island, N.Y.), 0.91 mM sodium pyruvate, 2.92 mM calcium lactate (Merck) and 3.05 mM glucose, pH 7.8 (Cheng *et al.*, 1986). Fertilisation medium (FM) was prepared by adding 2 mM caffeine (Merck) and 5.84 mM calcium lactate to the CM (M. Mattioli, personal communication), pH 7.4.

Oocyte recovery

Large White × Landrace prepubertal gilts weighing 80–95 kg were injected intramuscularly with 1250 IU pregnant mare serum gonadotrophin (PMSG; Intervet International, Boxmeer, Holland) followed 55 h later with 750 IU human chorionic gonadotrophin (hCG; Intervet). Freshly ovulated oocytes were obtained 42–46 h after hCG by flushing the oviducts during laparotomy as described by Coy (1992). A total of 795 oocytes were used. At the laboratory, the oocytes were washed twice in PBSDm (39°C) and classified in two groups. In the first group we included all those oocytes that retained the cumulus cell matrix and in the second group we included all those oocytes that were accidentally denuded during transport or manipulation but were in the cumulus cell matrix when they were recovered. Hence, they were denuded for a short time between recovery and fertilisation. Ten to fifteen cumulus-intact or denuded oocytes were transferred to each Petri dish (35 × 10 mm) containing FM at 39°C under 5% CO_2 in air. Both treatments were evaluated in each trial in experiment 1.

Sperm capacitation

Ejaculated boar spermatozoa were diluted immediately after collection, with a commercial extender preparation (MR-A; Martín *et al.*, 1983), to a final concentration of 3×10^7 spermatozoa/ml. The diluted spermatozoa were incubated for about 24 h at 15°C (Cheng *et al.*, 1986; Mattioli *et al.*, 1989). Before use, the spermatozoa were centrifuged at 100 *g* for 3 min to remove seminal plasma clots and other heavy particles; the supernatant was recovered and washed three times by centrifugation at 1200 *g* for 3 min. The first two times the pellet was resuspended in SWM, and the final pellet was resuspended by 2×10^8 spermatozoa/ml in 3 ml CM. Spermatozoa were incubated for 40–60 min at 39°C (Vázquez, 1991) in a tightly capped test tube. Only samples with progressive motility higher than 60% were used.

In vitro fertilisation and examination of oocytes

Experiment 1. A portion of the preincubated sperm suspension was added to the Petri dishes with FM containing the cumulus-intact or denuded oocytes so that the final concentration of spermatozoa at insemination was 6×10^5 cell/ml. The medium volume was 2 ml and the co-incubation time was 4 h.

Experiment 2. After classification, only cumulus-intact oocytes were used for this experiment. They were fertilised as in experiment 1, but the medium volume was 0.4 or 2 ml. Each trial included both treatments.

After 4 h of co-incubation the oocytes from both experiment 1 and 2 were transferred to fresh FM and adherent spermatozoa and cumulus masses were removed from the zona pellucida by pipetting (Mattioli *et al.*, 1989). Thereafter, the eggs were cultured for a further 12–14 h. The oocytes were then washed and fixed as whole mounts in ethanol:acetic acid (3:1 v/v) and stained in 1% lacmoid 24 h later (Chang, 1952). The fertilisation rate and the incidence and degree of polyspermy were evaluated by phase contrast microscopy. Oocytes at germinal vesicle (GV) stage were classified as immature. Oocytes with a broken oolemma or abnormal appearance of the cytoplasm were classified as degenerated. Oocytes with pronuclei (normally one or two) without any sperm tail were considered activated.

Table 1 Effect of cumulus cell presence on fertilisation of *in vivo* matured pig oocytes

Type of oocytes	Oocytes recovered	Healthy mature oocytes (%)	Fertilised oocytes (%)	Fertilisation range[a] (%)
Cumulus	231	178 (77.1)	123 (69.1)[b]	52–96
Denuded	282	203 (72.0)	109 (53.7)[b]	13–82

[a] Variation in the percentage of fertilisation after six trials.
[b] Groups with the same superscript are different ($p < 0.01$).

Table 2 Distribution of oocytes classified by the number of spermatozoa observed in the cytoplasm relative to the presence of cumulus cells

Type of oocytes	Healthy mature oocytes	Spz/oo[a] ($x \pm$ SEM)	Monospermic oocytes[b] (%)	Polyspermic oocytes[b] (%)		
				2 spz	3 spz	> 3 spz
Cumulus	178	1.52 ± 0.06[c]	75 (42.1)[c]	38 (21.3)[d]	6 (3.4)	4 (2.2)[b]
Denuded	203	2.33 ± 0.08[c]	43 (21.9)[c]	27 (13.3)[d]	15 (7.4)	24 (11.8)[b]

Spz, spermatozoa; oo, oocyte.
[a] Relative to number of fertilised oocytes.
[b] As a percentage of the total number of healthy mature oocytes.
[c] Groups with the same superscript in the same column are different ($p < 0.01$).
[d] Groups with the same superscript in the same column are different ($p < 0.05$).

Statistical analysis

Percentages of fertilisation, monospermy and polyspermic oocytes (2, 3 or > 3 spermatozoa per oocyte) were analysed by chi-squared tests. The numbers of spermatozoa per oocyte were subjected to analysis of variance. Comparisons between means were made by paired *t*-test.

Results

Experiment 1

Cumulus-intact oocytes had higher rates of fertilisation, with the range being higher in every trial (Table 1). The percentage of activated oocytes ranged from 1.1% to 4.9% in both groups.

The percentage of monospermic oocytes was significantly higher ($p < 0.01$) when cumulus cells, intercellular matrix and any other molecules added in the oviduct were present (Table 2). Relative to the fertilised oocytes, 61.0% and 39.4% of cumulus-intact and denuded oocytes were monospermic. The percentages of polyspermic oocytes with 2 ($p < 0.05$) and more than 3 ($p < 0.01$) spermatozoa inside the vitellus were significantly higher in denuded than in cumulus-intact oocytes.

Experiment 2

The volume of the co-incubation medium appeared not to affect the results of pig IVF, as is shown in Tables 3 and 4. However, if we compare the percentages of monospermic oocytes in every group as the number of monospermic oocytes in relation to the number of fertilised oocytes (rather than as the number of monospermic oocytes in relation to the total number of healthy mature oocytes) the percentage was significantly higher ($p < 0.05$) with 0.4 ml of medium (78%) than with 2 ml (57.5%). The mean number of spermatozoa per oocyte ($p < 0.05$) and the percentage of dispermic oocytes ($p < 0.01$) were significantly lower with 0.4 ml than with 2 ml. The percentage of activated oocytes ranged from 0.9% to 1.7% in both groups.

Table 3 Influence of the volume of co-incubation medium during IVF on the penetrability of *in vivo* matured oocytes

Medium volume (ml)	Oocytes recovered	Healthy mature oocytes (%)	Fertilised oocytes (%)	Fertilisation range[a] (%)
0.4	136	115 (84.6)	64 (55.6)	52–77
2	146	111 (76.0)	73 (65.8)	54–78

[a] Variation in the percentage of fertilisation in five trials.

Discussion

The presence of cumulus cells and intercellular matrix in the co-incubation medium increased the fertilisation and monospermy rates during IVF in pigs. Cumulus cells could exert their effect by reducing spermatozoa–oocyte collisions (Barros & Yanagimachi, 1972) or by improving the viability of the oocytes in the culture by continued synthesis of hyaluronic acid (Yang & Yanagimachi, 1989; Sato *et al.*, 1990). Oviductal glycoproteins which bind to the zona pellucida of newly ovulated pig eggs (Brown & Cheng, 1986) improve porcine IVF, including a reduction in the incidence of polyspermy (Nagai & Moor, 1990). These glycoproteins could reduce the simultaneous penetration of the zona pellucida or the enveloping cumulus matrix by two or more spermatozoa, thereby reducing the chances for polyspermic fertilisation (Hunter, 1991). These glycoproteins might also enter the perivitelline space (Verhage & Fazleabas, 1990) and facilitate a more synchronous exocytosis of cortical granule contents or increase physiological

Table 4 Influence of the volume of medium used during co-incubation on the number of spermatozoa observed in the ooplasm

Medium volume (ml)	Healthy mature oocytes	Spz/oo[a] ($x \pm$ SEM)	Monospermic oocytes[b] (%)	Polyspermic oocytes (%)		
				2 spz	3 spz	> 3 spz
0.4	115	1.28 ± 0.07^{c}	50 (43.5)	11 (9.6)[d]	2 (1.7)	1 (0.9)
2	111	1.50 ± 0.06^{c}	42 (37.8)	26 (23.4)[d]	4 (3.6)	1 (0.9)

Spz, spermatozoa; oo, oocyte.
[a] Relative to number of fertilised oocytes.
[b] As a percentage of the total number of healthy mature oocytes.
[c] Groups with the same superscript are different ($p < 0.05$).
[d] Groups with the same superscript column are different ($p < 0.01$).

responses of the zona pellucida to cortical granule material (Barros & Yanagimachi, 1971; Yang & Yanagimachi, 1989). Since all our oocytes were recovered from the oviducts, the oviductal glycoproteins were always present. Our comparatively low rates of polyspermy are consistent with all previous hypotheses regarding the effects of oviduct glycoproteins on the zona pellucida. The glycoproteins might also stabilise the sperm plasma membrane and reduce the number of spermatozoa capacitated simultaneously, as hypothesized by Hunter (1991).

Alternatively, cumulus cells might have receptors for the oviductal glycoproteins or other oviductal factor(s), or the oviductal secretions might influence the intercellular matrix. This might also explain the different appearance of the cumulus recovered from the oviduct compared with that from the follicular oocytes just before ovulation (Braden, 1962). Binding of the glycoproteins could facilitate the entry of spermatozoa into the cumulus and explain the higher rates of fertilisation in our study and that of Kikuchi *et al.* (1991). On the other hand, the spermatozoa could induce some change which delayed or made more difficult the entry of other spermatozoa, perhaps by means of the enzymes released during the acrosome reaction, facilitating monospermic penetration. Although the presence of cumulus cells might also benefit capacitation or the acrosome reaction (Gwatkin *et al.*, 1972; Yanagimachi, 1988), such an effect does not explain the reduced rate of polyspermy we observed.

Different workers have used 2 ml, 0.4 ml, 0.2 ml and 0.1 ml for porcine IVF, with variable results, but they have not studied the effects of changing the volume of medium. Our results show no differences in fertilisation rates with 0.4 ml or 2 ml of medium. Although some parameters related to monospermy appeared to be improved by using the reduced volume, we do not have enough data to explain the mechanism.

In conclusion, the continued presence of cumulus cells and the intercellular matrix increased the rates of fertilisation and monospermy in porcine IVF. The volume of gamete co-incubation medium seemed to influence some parameters related to monospermy. This volume effect should be studied further.

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