In-vitro penetration of pig oocytes matured in culture by frozen–thawed ejaculated spermatozoa

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Summary. Pig oocytes matured in culture were inseminated with frozen–thawed ejaculated spermatozoa without preincubation in modified tissue culture medium (TCM) 199. High penetration rates (85–89%) and increased incidence of polyspermy were obtained at $25\text{--}100 \times 10^6$ spermatozoa/ml. Wide variation in penetration rates (16–89%) was observed in oocytes inseminated in medium containing 5mM caffeine and at $25\text{--}50 \times 10^6$ spermatozoa/ml obtained from 6 boars, regardless of sperm motility. At $25\text{--}50 \times 10^6$ spermatozoa/ml, penetration rates of oocytes were dependent upon the concentration of caffeine in the medium: there was no penetration without caffeine, but penetration was highest (89%) with 5mM caffeine. None of the oocytes was penetrated in the medium supplemented with heparin at 5–40 µg/ml. When heparin was included in the medium with 5mM caffeine, it inhibited the efficacy of caffeine to promote sperm penetration of oocytes.

Keywords: in-vitro fertilization; oocyte; pig; frozen ejaculated sperm

Introduction

Successful in-vitro fertilization of pig oocytes matured *in vivo* or *in vitro* by fresh ejaculated or epididymal spermatozoa has been reported (Iritani *et al.*, 1978; Nagai *et al.*, 1984, 1990; Cheng, 1985; Hamano & Toyoda, 1986; Yoshida, 1987; Naito *et al.*, 1988; Hamano *et al.*, 1989; Mattioli *et al.*, 1989; Yoshida *et al.*, 1990). Nagai *et al.* (1988) have reported that pig oocytes matured *in vivo* and *in vitro* can be penetrated *in vitro* by frozen epididymal spermatozoa. Repeated use of spermatozoa with the same characteristics for in-vitro fertilization would be a great advantage for fundamental research on fertilization mechanisms. Epididymal spermatozoa are usually obtained after boars have been killed; it is therefore not possible to use spermatozoa collected repeatedly from the same boar. This problem would be solved if techniques for fertilizing pig oocytes *in vitro* by frozen ejaculated spermatozoa were developed. The present study was designed to develop such techniques.

Materials and Methods

Culture medium

The basic medium, designated TCM-199B, for the manipulation of spermatozoa and oocytes was TCM-199 (with Earle's salts, Gibco Lab., USA) supplemented with 3.05mM D-glucose, 2.92mM calcium lactate, 0.91mM sodium pyruvate, 75 µg potassium penicillin G/ml, 50 µg streptomycin sulphate/ml and 10% (v/v) heated fetal calf serum (FCS). This medium was essentially the same as that described by Cheng (1985), except that FCS was added at 10% rather than 12% and that penicillin and streptomycin, rather than dibekacin, were used as the antibiotics.

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Culture of follicular oocytes

Ovaries were collected from maturing gilts at a local slaughterhouse and returned to the laboratory in 0.9% NaCl (w/v) containing 75 µg potassium penicillin G/ml and 50 µg streptomycin sulphate/ml at 37–39°C within 1 h. Oocytes were aspirated through an 18-gauge needle into a disposable 1-ml syringe from follicles 2–5 mm in diameter. The collected oocytes were washed 4 times with the maturation medium, TCM-199B supplemented with 10 iu pregnant mares' serum gonadotrophin/ml, 10 iu human chorionic gonadotrophin/ml and 1 µg oestradiol-17β/ml (Sigma Chemical Co., St Louis, MO, USA). Ten oocytes with compact cumulus masses were transferred to a droplet of 100 µl of maturation medium covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm) and cultured for 36 h at 39°C in 5% CO₂ in air. In a preliminary experiment, of 78 oocytes with an expanded cumulus mass, 5 were still at the germinal vesicle stage, but 18 (23%) and 55 (71%) were at the stages of metaphase to telophase I and metaphase II, respectively. Since it was difficult to distinguish matured from immature oocytes without removing cumulus cells, cultured oocytes with an expanded cumulus mass were used for in-vitro fertilization. Five oocytes were washed twice and placed into 50 µl TCM-199B with different concentrations of caffeine and sodium benzoate (Sigma Chemical Co.) and/or pig intestinal mucosal heparin (176 United States Pharmacopoeia units/ml; Sigma Chemical Co.), which had been covered with paraffin oil in a culture dish. The dishes were kept in a CO₂ incubator (5% CO₂ in air at 39°C) for ~30 min until spermatozoa were added for fertilization.

Sperm preparation

The sperm-rich portion of the ejaculated semen collected from boars by the glove method and filtering through double gauze was kept at 15°C for 2–3 h. Semen samples were then diluted 1:2 with Hülseberger VIII diluent (Richter *et al.*, 1975) at 15°C and centrifuged for 15 min at 300 g. The supernatant was discarded and spermatozoa were resuspended in Beltsville F5 extender (Pursel & Johnson, 1975) to give 10×10^8 cells/ml. After cooling to 5°C over 1 h, the sperm suspensions were diluted with the same volume of BF-5 solution containing 2% (v/v) glycerol. The sperm suspensions were frozen in 0.1 ml pellets on solid CO₂ and stored in liquid nitrogen until required for in-vitro fertilization. Frozen spermatozoa (8 pellets) were thawed in 2 ml TCM-199B at 37°C for ~1 min. After thawing, 6–8 ml TCM-199B was added and spermatozoa were washed 3 times by centrifugation at 550 g for 5 min each time. The spermatozoa were resuspended in TCM-199B to give $2\text{--}200 \times 10^6$ cells/ml. Before centrifugation (just after thawing) and after centrifugation (at insemination), the percentage of spermatozoa with progressive forward motility was examined under a phase-contrast microscope on a warm plate at 37°C.

In-vitro fertilization and examination of oocytes

A 50-µl sample of final sperm suspension was introduced into 50 µl of TCM-199B with different concentrations of caffeine and/or heparin that included the oocytes.

At 14–16 h after insemination (5% CO₂ in air at 39°C), oocytes were mounted, fixed for 48 h in 25% (v/v) acetic alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of ×200 or ×400. Oocytes were considered as penetrated when they had a swollen sperm nucleus(ei) and/or a male pronucleus(ei) and corresponding sperm tail(s) in the cytoplasm. Oocytes with >2 pronuclei and a clear second polar body, even without a sperm tail, were also considered as penetrated.

Experiment 1. The effect of sperm concentration on in-vitro penetration of oocytes was examined using spermatozoa, obtained from boar D26, in TCM-199B containing 5mM caffeine.

Experiment 2. The abilities of spermatozoa from 6 boars to penetrate oocytes *in vitro* were compared. The final concentrations of spermatozoa at fertilization were $25\text{--}50 \times 10^6$ cells/ml in TCM-199B with 5mM caffeine.

Experiment 3. The effect of concentration of caffeine in TCM-199B on in-vitro penetration of oocytes was examined using spermatozoa from boar D26 at $25\text{--}50 \times 10^6$ cells/ml.

Experiment 4. It has been reported that in cattle caffeine and heparin have synergistic effects on inducing capacitation of frozen bull spermatozoa (Niwa & Ohgoda, 1988); the possible occurrence of such an effect in fertilization of pig oocytes *in vitro* was investigated. Spermatozoa from boars D26 and W860 were used at $25\text{--}50 \times 10^6$ cells/ml.

Results

Experiment 1

Few oocytes were penetrated at sperm concentrations of $1\text{--}6 \times 10^6$ cells/ml (Table 1), but high penetration rates (85–89%) and an increased incidence of polyspermy were obtained with $>25 \times 10^6$ spermatozoa/ml. The proportions of penetrated oocytes with male and female pronuclei were low (11–38%) and not correlated with sperm concentration. The mean number of penetrated spermatozoa per oocyte increased with increasing sperm concentration.

Table 1. Penetration *in vitro* of pig oocytes by frozen-thawed ejaculated spermatozoa (from boar D26) at different sperm concentrations in the presence of 5mM caffeine

Sperm concentration ($\times 10^{-6}$ /ml)	No. of trials	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)	Mean no. of spermatozoa in penetrated oocytes
			Total (%)	With swollen sperm head	With male and female pronuclei		
1	6	88	0 (0)	0	0	—	—
2	3	50	0 (0)	0	0	—	—
3	5	75	3 (4)	2	1	0 (0)	1.0
6	5	83	10 (12)	7	3	4 (40)	1.6
12	5	79	34 (43)	21	13	18 (53)	2.0
25	5	75	66 (88)	41	24	41 (62)	2.3
50	4	53	45 (85)	40	5	30 (67)	2.6
100	6	90	80 (89)	60	20	56 (70)	3.1

Experiment 2

The percentages of motile spermatozoa at fertilization were low (5–20%) in all boars used (Table 2). In general, the motility of spermatozoa not directly associated with the oocyte-cumulus complex in the medium decreased rapidly after insemination. Spermatozoa from boars D26, W860 and L612 showed similar motility (20%), but differed greatly in penetration rate (19–89%) of oocytes. The proportions of oocytes penetrated by spermatozoa from boars D1, D49 and D125, which had motilities of 10, 10 and 5%, respectively, were comparatively low.

Table 2. Penetration *in vitro* of pig oocytes by frozen-thawed ejaculated spermatozoa ($25\text{--}50 \times 10^6$ /ml) obtained from 6 boars in the presence of 5mM caffeine

Boar no.	No. of trials	Percentage of motile spermatozoa at fertilization	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
				Total (%)	With swollen sperm head	With male and female pronuclei	
D1	8	10	91	48 (53)	27	21	18 (38)
D26	3	20	44	39 (89)	21	18	26 (67)
D49	3	10	38	18 (47)	13	5	6 (33)
D125	6	5	75	12 (16)	12	0	0 (0)
W860	4	20	50	43 (86)	19	24	20 (47)
L612	4	20	48	9 (19)	7	2	1 (11)

Experiment 3

None of the oocytes was penetrated when caffeine was not included in the medium (Table 3). The highest proportion (89%) of oocytes was penetrated with 5.0mM caffeine ($P < 0.05$), but, at this caffeine concentration, incidence of polyspermy was also high (67%).

Experiment 4

None of the oocytes was penetrated at any concentrations of heparin in the medium without caffeine (Table 4). With 5mM caffeine in the medium, the proportion of oocytes penetrated decreased as the concentration of heparin increased; the penetration of oocytes was completely inhibited when the medium was supplemented with 40 μ g heparin/ml.

Table 3. Effect of concentration of caffeine in the medium on penetration *in vitro* of pig oocytes by frozen-thawed ejaculated spermatozoa ($25\text{--}50 \times 10^6/\text{ml}$ from boar D26)

Concentration of caffeine (mM)	No. of trials	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
			Total (%)	With swollen sperm head	With male and female pronuclei	
0.0	3	41	0 (0)	0	0	—
2.5	3	40	28 (70)	19	9	18 (64)
5.0	3	44	39 (89)*	21	18	26 (67)
10.0	3	46	30 (65)	23	7	12 (40)
20.0	3	42	4 (9)	4	0	0 (0)

* $P < 0.05$ compared with the values at all other concentrations of caffeine (χ^2 test).

Table 4. Effect of heparin in the medium with or without caffeine on penetration *in vitro* of pig oocytes by frozen-thawed ejaculated spermatozoa ($25\text{--}50 \times 10^6/\text{ml}$)

Concentration of heparin ($\mu\text{g}/\text{ml}$)	With caffeine (5mM)*				Without caffeine†		
	No. of trials	No. of oocytes		No. of polyspermic oocytes (%)	No. of trials	No. of oocytes	
		Examined	Penetrated‡ (%)			Examined	Penetrated (%)
0	4§	50	19 + 24 (86)	20 (47)	3¶	41	0 (0)
5	3	35	9 + 13 (63)	10 (46)	—	—	—
10	3	40	15 + 8 (56)	11 (48)	3	39	0 (0)
20	3	46	9 + 7 (35)	3 (19)	3	49	0 (0)
30	3	40	6 + 4 (25)	3 (30)	3	50	0 (0)
40	3	41	0 + 0 (0)	—	3	52	0 (0)

*Spermatozoa from boar W860.

†Spermatozoa from boar D26.

‡The first figure denotes the number of oocytes with swollen sperm head(s); the second denotes the number with male and female pronuclei.

§Data from boar W860, Table 2.

¶Data from boar D26, Table 3.

Discussion

Nagai *et al.* (1988) reported that, in contrast to fresh ejaculated or frozen epididymal spermatozoa, no frozen ejaculated spermatozoa penetrated pig oocytes matured in culture. In their study, the spermatozoa were preincubated for 4 h before insemination, which resulted in great reduction of sperm motility. The reduced motility of frozen ejaculated spermatozoa after preincubation was also reported by Clarke & Johnson (1987). Since, in the present study, percentages of sperm motility of frozen-thawed ejaculated spermatozoa from 6 boars were low (5–20%), spermatozoa were used without preincubation for in-vitro fertilization of oocytes. Although further and rapid reduction of sperm motility was observed in spermatozoa not directly associated with the oocyte–cumulus complex, the present results clearly show that frozen ejaculated boar spermatozoa can penetrate oocytes matured in culture. This result may indicate that spermatozoa penetrating the cumulus mass before complete loss of motility can maintain their ability to undergo capacitation and to penetrate oocytes. The necessity of cumulus cells for frozen boar epididymal spermatozoa to complete capacitation is suggested by Nagai *et al.* (1988).

The present results indicate that sperm concentrations at fertilization are important for penetration of oocytes with frozen ejaculated spermatozoa. High penetration rates (85–89%) were obtained with spermatozoa from boars D26 and W860 at $25\text{--}100 \times 10^6$ spermatozoa/ml (Tables 1 and 2), but these concentrations are not necessarily optimum for spermatozoa from other boars. For example, spermatozoa from boar L612 showed low penetrability (19%). Such variation occurred regardless of sperm motility because the frozen ejaculated spermatozoa from these 3 boars showed the same percentage (20%) of motility at fertilization. It is not clear whether the optimal sperm concentration for fertilization of oocytes or the ability of spermatozoa to penetrate oocytes differs among boars. On the other hand, if sperm concentration had been increased in frozen semen or samples from boars D1, D49 and D125, which showed less motility (5–10%) and lower penetrability (16–53%), penetration rates of oocytes might have increased, but this was not examined in the present study.

Several investigators have used medium supplemented with 2mM caffeine for in-vitro fertilization of pig oocytes (Hamano & Toyoda, 1986; Yoshida, 1987; Naito *et al.*, 1988; Nagai *et al.*, 1988; Hamano *et al.*, 1989). It has been reported that caffeine enhances and prolongs motility of bull and boar spermatozoa (Garbers *et al.*, 1971, 1973) and that the treatment of frozen-thawed bull spermatozoa with caffeine results in increasing penetration rates *in vitro* of oocytes (Niwa *et al.*, 1988; Ohgoda *et al.*, 1988). Fraser (1979) has reported that caffeine may accelerate the rate of sperm capacitation in mice. Since, in the present study, penetration rates of pig oocytes *in vitro* were dependent upon the concentration of caffeine supplemented to the medium (resulting in no penetration without caffeine and the highest penetration rate (89%) at 5mM), it appears that caffeine has an essential role(s) for either inducing capacitation of spermatozoa or penetration of oocytes.

Heparin has been known to stimulate capacitation of bull spermatozoa *in vitro* (Parrish *et al.*, 1988). The synergistic effect of caffeine and heparin in inducing capacitation of frozen-thawed bull spermatozoa, resulting in increased penetration rates *in vitro* of oocytes matured in culture, has also been reported (Niwa & Ohgoda, 1988). In the present study, however, heparin did not support in-vitro penetration of pig oocytes with frozen ejaculated spermatozoa, but inhibited the efficacy of caffeine to promote penetration of oocytes. Although it is difficult to explain why species difference in heparin effects occur, different functions of heparin have been reported in hamsters, in which an acrosome reaction, rather than capacitation of spermatozoa, appears to be induced by heparin (Meizel & Turner, 1986).

In the present study, the proportion of penetrated oocytes with male and female pronuclei were low (0–59%) and the incidence of polyspermy was high. The poor formation of male pronuclei (Motlik & Fulka, 1974; Nagai *et al.*, 1984, 1988; Mattioli *et al.*, 1988; Naito *et al.*, 1988) and high incidence of polyspermy (Nagai *et al.*, 1984; Cheng, 1985; Mattioli *et al.*, 1988) in pig oocytes matured in culture have been repeatedly reported by many workers. Although abnormal formation of male pronuclei can result from incomplete maturation *in vitro*, it appears that the high incidence of polyspermy is not due entirely to abnormal maturation of oocytes, because pig oocytes matured *in vivo* are also penetrated by high numbers of spermatozoa after in-vitro fertilization (Cran & Cheng, 1986). Since the requirements of follicle somatic cells (Mattioli *et al.*, 1988; Moor *et al.*, 1990) and pig follicular fluid (Naito *et al.*, 1988) for normal in-vitro maturation of pig oocytes have recently been clarified, it is important to develop procedures that prevent polyspermy during in-vitro fertilization of pig oocytes (Nagai & Moor, 1990).

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