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Reduction of the incidence of polyspermic penetration into porcine oocytes by pretreatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine

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Abstract

To reduce the incidence of polyspermic penetration, the effects of transient exposure of washed fresh spermatozoa to caffeine in a brief co-culture *in vitro* fertilization (IVF) system were examined. A pretreatment effect of spermatozoa with adenosine was also examined. When 5 mmol caffeine/l was supplemented during periods of co-culture and additional culture periods until 8 h after insemination, a shortened co-incubation period of gametes (30 denuded oocytes in 100 μl modified Medium 199-suspended spermatozoa at 2.5×10^5 sperm/ml) from 30 to 5 min increased the monospermy rate in total mature oocytes examined. The number of spermatozoa binding to the zona surface was significantly lower in oocytes co-cultured for 5 min (33.1 ± 2.2) than 8 h (207.6 ± 13.7). A limited exposure of gametes to 5 mmol caffeine/l only during a transient co-culture period for 5 or 30 min significantly reduced the mean number of sperm cells that penetrated into the oocyte. Transient exposure of spermatozoa to caffeine for only 5 min increased the percentage of capacitated cells but not acrosome-reacted cells, as compared with a whole exposure treatment. Furthermore, preincubation of spermatozoa with 10 μmol adenosine/l for 90 min increased both the incidence of capacitated cells and the monospermy rate and consequently decreased the number of sperm cells that penetrated into the oocyte. In conclusion, these results have demonstrated that a new transient co-incubation IVF system, in which denuded oocytes are co-cultured with spermatozoa in medium containing caffeine for 5 to 30 min and then continuing the culture in caffeine-free medium, will reduce the incidence of polyspermic penetration. Preincubation of fresh spermatozoa with adenosine before the transient co-incubation IVF can also improve the monospermy rate. Furthermore, asynchrony in the morphology of sperm nuclei in polyspermic oocytes was reduced by the pretreatment with adenosine and a brief exposure to caffeine.

Reproduction (2004) **128** 789–800

Introduction

A high incidence of polyspermic penetration is still a major obstacle to the production of normal porcine embryos *in vitro* (Funahashi & Day 1997, Day 2000, Abeydeera 2002, Coy & Romar 2002, Funahashi 2003) and is probably due to a slow zona reaction and simultaneous sperm penetration (Sun *et al.* 1992, Wang *et al.* 1998). Some progress has been made towards overcoming this problem by efforts to increase the quality of *in vitro* matured oocytes and to decrease simultaneous penetration by regulating the sperm–zona pellucida binding (see Funahashi 2003 for review). Furthermore, some unique porcine *in vitro* fertilization (IVF) systems to allow a gradient arrival of actively motile spermatozoa to the surface of oocytes, including the ‘climbing-over-the-wall’ method (Funahashi & Nagai 2000) and the ‘straw IVF’ method (Li *et al.* 2003), have also been developed and partially succeeded in improving the efficiency of normal fertilization. However, there seems to be much room for further improvement in the efficiency of porcine IVF systems, especially by reducing the simultaneous initiation of acrosome reaction which triggers a sperm race for penetration (Funahashi 2003). In a majority of porcine IVF systems (Cheng *et al.* 1986, Yoshida 1987, Nagai *et al.* 1988, Mattioli *et al.* 1989), spermatozoa and oocytes have

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.00295
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been co-cultured in the presence of caffeine for several hours or overnight. Caffeine is generally thought of as a molecule that inhibits cyclic nucleotide phosphodiesterase, resulting in an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) (Casillas & Hoskins 1970) and induction of capacitation and spontaneous acrosome reactions of boar spermatozoa (Funahashi *et al.* 2000*b,c*). If caffeine is added to IVF medium, in fact, sperm penetration is achieved by 6 h after insemination, accompanied by an increase in polyspermic penetration (Abeydeera & Day 1997, Shimada *et al.* 2000, Funahashi & Nagai 2001). Since acrosome-intact boar spermatozoa are known to initiate binding to the porcine zona pellucida (Fazeli *et al.* 1997), a relatively longer duration of co-culture of gametes in the presence of caffeine may increase the chance for more spermatozoa to bind to the zona pellucida, to induce the capacitation/acrosome reaction and to achieve penetration within a narrow window. Although it was recently demonstrated that a shortened period of sperm–oocyte co-culture during IVF significantly reduced the incidence of polyspermic penetration into porcine oocytes matured *in vitro* (Grupen & Nottle 2000, Gil *et al.* 2004), these gametes were still exposed to caffeine for more than 5 h after co-culture. Therefore, not only a reduced period of co-culture of gametes, such as the two-step IVF procedure (Grupen & Nottle 2000, Gil *et al.* 2004), but also a short exposure of spermatozoa to caffeine may decrease the number of spermatozoa initiating penetration of the zona pellucida and consequently reduce the incidence of simultaneous multiple sperm penetration.

Furthermore, we recently demonstrated that both adenosine and fertilization-promoting peptide (FPP, which is a tripeptide, pGlu-Glu-ProNH_2) induce capacitation of both fresh and frozen–thawed boar spermatozoa *in vitro* but prevent a spontaneous acrosome reaction via the adenylyl cyclase/cAMP pathway, even in the presence of caffeine (Funahashi *et al.* 2000*b,c*). Supplementation with adenosine and FPP during co-culture of oocytes with frozen–thawed spermatozoa increased the proportion of monospermic penetration (Funahashi *et al.* 2000*c*). Although utilization of adenosine or FPP in combination with the current transient co-culture IVF system may be expected to reduce the polyspermic penetration rate further, the efficacy of adenosine or FPP to achieve a successful incidence of monospermic penetration in an IVF system with fresh boar spermatozoa has not been demonstrated.

In the present study, to enhance the concept of a brief gamete co-culture system (Grupen & Nottle 2000, Gil *et al.* 2004), the effect of a transient exposure of fresh boar spermatozoa to caffeine on the incidence of monospermic penetration into porcine oocytes was examined. The effect of preincubation of fresh spermatozoa with adenosine on monospermic penetration was also examined in the current transient exposure system.

Materials and Methods

Chemicals and culture media

KCl, KH_2PO_4, $MgCl_2$, $CaCl_2$, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical Co. Ltd (Osaka, Japan). NaCl and paraffin liquid were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Other chemicals were purchased from Sigma Aldrich Japan K K (Tokyo, Japan). As a caffeine reagent, a mixture of caffeine and sodium benzoate at equal weight (C-4144; Sigma) was used in this study.

The medium used for the collection of oocyte–cumulus complexes and washing was modified TL-HEPES-PVA medium composed of 114 mmol NaCl/l, 3.2 mmol KCl/l, 2 mmol $NaHCO_3$/l, 0.34 mmol KH_2PO_4/l, 10 mmol Na-lactate/l, 0.5 mmol $MgCl_2$/l, 2 mmol $CaCl_2$/l, 10 mmol HEPES/l, 0.2 mmol Na-pyruvate/l, 12 mmol sorbitol/l, 0.1% (w/v) polyvinylalcohol, 25 μg gentamicin/ml and 65 μg potassium penicillin G/ml. The basic maturation medium (OMM37) used was bovine serum albumin (BSA)-free North Carolina State University 37 (NCSU-37) medium (Petters & Wells 1993) supplemented with 0.6 mmol cysteine/l, 5 μg insulin/ml and 10% (v/v) porcine follicular fluid (Funahashi *et al.* 1997). The basic medium for fertilization *in vitro* was modified Medium 199 (m-M199), which is Medium 199 with Earle's salts (GIBCO; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 3.05 mmol glucose/l, 2.92 mmol hemi-calcium lactate/l, 0.91 mmol sodium pyruvate/l, 1200 mmol sorbitol/l, 25 μg gentamicin/ml, 65 μg potassium penicillin G/ml and 0.4% (w/v) BSA (Sigma). The medium used as semen diluent was the modified Modena solution prepared with 152.64 mmol glucose/l, 23.46 mmol sodium citrate/l, 11.9 mmol $NaHCO_3$/l, 6.99 mmol EDTA-2Na/l, 46.66 mmol Tris/l, 15.10 mmol citric acid/l and 10 mg gentamicin sulfate/l. All media without modified TL-HEPES-PVA and modified Modena solution were equilibrated under paraffin liquid at 39 °C in an atmosphere of 5% CO_2 in air overnight prior to incubation with oocytes. Porcine follicular fluid was prepared from antral follicles (3–6 mm in diameter) as described previously (Funahashi *et al.* 1994).

Preparation and culture of cumulus–oocyte complexes

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory within 2 h at 28–33 °C. Cumulus–oocyte complexes were aspirated through an 18 gauge needle into a disposable 10 ml syringe from antral follicles (3–6 mm in diameter) on the surface of ovaries, washed three times with modified TL-HEPES-PVA medium, and then collected in fresh modified TL-HEPES-PVA medium at room temperature (Wongsrikeao *et al.* 2004). This process was completed within 90 min. Oocytes were matured in an *in vitro* maturation (IVM) system that has been reported to produce blastocysts and piglets efficiently following IVF and embryo

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transfer (Funahashi *et al.* 1997). Briefly, 50 cumulus–oocyte complexes with uniform ooplasm and a compact cumulus cell mass were washed three times with OMM37 supplemented with 1 mmol dibutyryl cAMP/l, 10 IU eqine chorionic gonadotropin (eCG)/ml and 10 IU human chorionic gonadotropin (hCG)/ml, and subsequently cultured in 500 μl of the same medium covered with paraffin oil for 20 h at 39 °C in an atmosphere of 5% CO_2 in air. The complexes were then transferred to 500 μl OMM37 (without dibutyryl cAMP, eCG and hCG) after washing three times with the same medium. The complexes were cultured for an additional 24 h (Funahashi *et al.* 1994). After culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and washed three times with m-M199.

Preparation of fresh boar spermatozoa and ICF

Semen-rich fractions (30–50 ml) were collected from four Berkshire boars by the glove-hand method at a local experimental station and diluted four times with modified Modena solution. The diluted semen samples from different sperm sources in each replication were transported to the laboratory at 26–32 °C within 2 h after collection. After washing once by centrifugation at 750 ***g*** for 3 min, spermatozoa were re-suspended at a concentration of 1×10^8 cells/ml in modified Modena solution containing 5 mmol cysteine/l and 20% (v/v) boar seminal plasma. Diluted sperm suspension was cooled down from room temperature to 15 °C for 4 h and then kept overnight at the same temperature. Just before use, stored spermatozoa were placed at room temperature for 15–20 min, washed three times by centrifugation at 750 ***g*** for 3 min with modified TL-HEPES-PVA solution and then re-suspended at a concentration of 5×10^5 cells/ml in m-M199 supplemented with or without adenosine.

Just after dilution or preincubation for 90 min at 5×10^5 cells/ml in m-M199 containing or not containing adenosine, 50 μl diluted sperm suspension was inseminated in the same volume of m-M199 containing or not containing 10 mmol caffeine/l. Thirty denuded oocytes were co-cultured with spermatozoa (at a final sperm concentration of 2.5×10^5 cells/ml) in 100 μl droplets of m-M199 containing 5 mmol caffeine/l (n-M199–caffeine) under paraffin oil for 5–30 min. After co-culture, the oocytes were gently washed once with n-M199–caffeine or m-M199, transferred to a fresh 100 μl droplet of the same medium without spermatozoa and the culture was continued at 39 °C in an atmosphere of 5% CO_2 in air until 8 h after insemination. Since sperm penetration occurs within 6 h after insemination in the presence of caffeine or adenosine (Funahashi & Nagai 2001), the cultured eggs were fixed at 8 h after insemination, stained with 1% (w/v) orcein, and examined at 200× and 400× magnification. Oocytes were designated as penetrated when they had at least one sperm head, a decondensed sperm nucleus, or a male pronucleus and corresponding sperm tail in the vitellus.

Chlortetracycline (CTC) fluorescence assessment of spermatozoa

The methods used for CTC analysis were essentially those described previously (Funahashi *et al.* 2000*b*) with minor modifications. Briefly, 8 μl of 100 μg Hoechst bis-benzimide 33258/ml (Sigma) was added to 792 μl sperm suspension. After gentle mixing, each suspension was incubated for 3 min at room temperature in the dark, then layered onto 4 ml of 3% (w/v) polyvinylpyrolidone (PVP-40) in TL-HEPES-PVA and centrifuged at 750 ***g*** for 3 min. The pelleted spermatozoa were resuspended in 45 μl TL-HEPES-PVA and 45 μl of this suspension was added to 45 μl CTC solution, containing 750 μmol CTC/l, 5 mmol cysteine/l, 130 mmol NaCl/l and 20 mmol Tris/l (pH 7.8). Sperm cells were fixed by adding 8 μl 12.5% (w/v) paraformaldehyde in 0.5 mol Tris–HCl/l (pH 7.4). The CTC solution was prepared daily. Slides were prepared by placing 10 μl of the fixed sperm suspension on a slide and one drop of 0.22 mol 1,4-diazabicyclo[2.2.2]octane/l dissolved in glycerol:TL-HEPES-PVA (9:1) which was then carefully mixed in order to retard fading of the fluorescence. A coverslip was added and sealed with colorless nail varnish. Spermatozoa were assessed under a phase-contrast microscope, equipped with epifluorescent optics, on the same day. Each cell was first observed under u.v. illumination (excitation at 330–380 nm, emission at 420 nm) to determine the live/dead status; the sperm cells showing bright blue staining of the nucleus were considered to be dead and were not counted. More than 100 live sperm were then examined under blue-violet illumination (excitation at 400–440 nm, emission at 470 nm) and classified according to CTC staining patterns. The three fluorescent staining patterns identified were: F, with uniform fluorescence over the whole sperm head; B, with a fluorescence-free band in the post-acrosome region; and AR, with almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment.

Experimental design

The experimental design is schematically represented in Fig. 1.

In the first experiments, to determine if the beneficial effect of a shortened co-culture period of oocytes on the monospermic penetration rate was observed even with fresh spermatozoa, the co-culture duration was shortened from 30 to 5 min. After co-culture with fresh spermatozoa in n-M199–caffeine for 5, 10, 15 and 30 min at 39 °C in an atmosphere of 5% CO_2 in air, 30 oocytes were gently washed once with n-M199–caffeine, transferred to a fresh 100 μl droplet of the same medium without spermatozoa and the culture continued until 8 h after insemination.

In the second experiment, the relation between the duration of co-culture with fresh spermatozoa and the number of sperm binding to the zona pellucida was determined. At 5 min and 8 h of co-culture of gametes in

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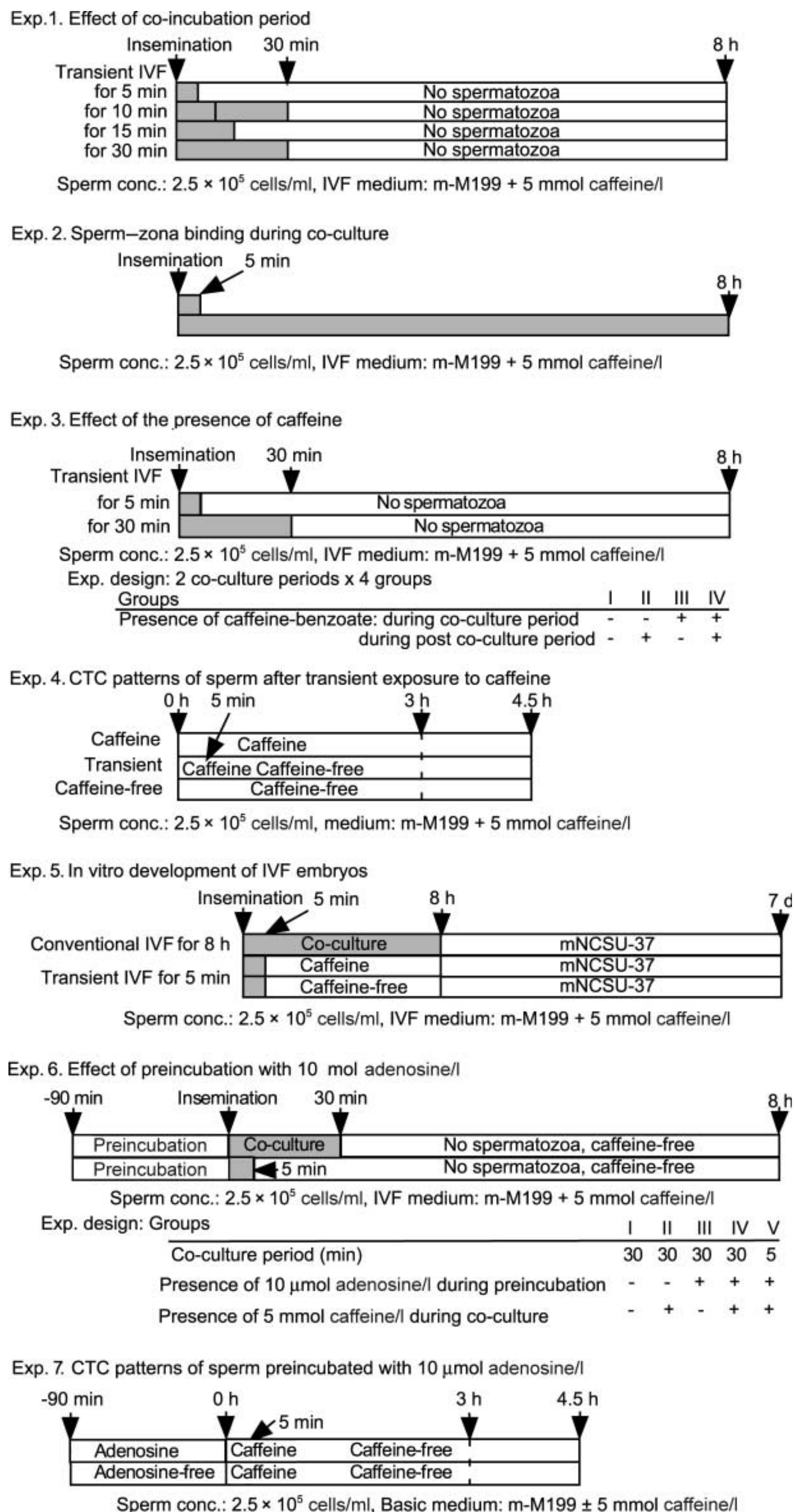

Figure 1 Schematic representation of experimental (Exp.) design, showing the different treatments, sperm concentrations and durations.

n-M199–caffeine at 39 °C in an atmosphere of 5% CO_2 in air, the oocytes were washed once with TL-HEPES-PVA and fixed with 3% paraformaldehyde in TL-HEPES-PVA for 30 min at room temperature. According to a previous report (Katayama *et al.* 2002), the oocytes were processed and stained with 20 µg/ml lectin from Archis hypogaea (peanut) conjugated fluorescein isothiocyanate (FITC-PNA) and 400 µl/ml propidium iodide. The number of spermatozoa binding to the surface of zona pellucida was observed by using a confocal laser scanning microscope (MRC1024; Nippon Bio-Rad Laboratories, Tokyo, Japan).

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In the third experiment, the effect of the presence of caffeine during co-culture and the post co-culture periods was examined. After co-culture with fresh spermatozoa in m-M199 or n-M199–caffeine for 5 or 30 min at 39 °C in an atmosphere of 5% CO_2 in air, oocytes were gently washed once with sperm-free m-M199, transferred to a 100 μl droplet of fresh n-M199–caffeine or m-M199 and the culture continued until 8 h after insemination.

In the fourth experiment, the effect of transient exposure to caffeine on sperm function was examined. Fresh spermatozoa were cultured in n-M199–caffeine for 5 min at 39 °C in an atmosphere of 5% CO_2 in air, and then culture continued in m-M199 after washing by centrifugation twice at 750 ***g*** for 3 min. At 3 and 4.5 h after the start of culture, CTC patterns of the sperm cells were compared with those of spermatozoa cultured in m-M199 or n-M199–caffeine for the whole duration of 3 or 4.5 h.

In the fifth experiment, *in vitro* development of oocytes fertilized in a transient IVF system was compared with that of oocytes fertilized in a conventional IVF system. After transient co-culture with spermatozoa at a concentration of 2.5×10^5 cells/ml in n-M199–caffeine for 5 min at 39 °C in an atmosphere of 5% CO_2 in air and gentle washing, the oocytes were transferred to m-M199 or n-M199–caffeine. At 8 h after insemination, the oocytes in transient and conventional IVF systems were moved again to modified NCSU-37 medium supplemented with 0.4% (w/v) BSA, 0.6 mmol cysteine/l and 5 μg insulin/ml and cultured for 7 days.

In the sixth experiment, the effect of preincubation of spermatozoa with adenosine before insemination was determined by using the transient co-incubation IVF system which was developed in the third experiment. Before insemination, spermatozoa were preincubated at a concentration of 5×10^5 cells/ml in the absence or presence of 10 μmol adenosine/l in m-M199 in an atmosphere of 5% CO_2 in air at 39 °C for 90 min. After co-culture with the spermatozoa (at a final concentration of 2.5×10^5 cells/ml) in m-M199 or n-M199–caffeine for 5 or 30 min, oocytes were gently washed once with sperm-free m-M199, transferred to a 100 μl droplet of fresh m-M199 and the culture continued until 8 h after insemination.

In the final (seventh) experiment, the effect of preincubation with adenosine on sperm function was determined. Fresh spermatozoa at a concentration of 5×10^5 cells/ml were precultured in m-M199 supplemented with or without 10 μmol adenosine/l for 90 min and then cultured in n-M199–caffeine for 5 min (at a concentration of 2.5×10^5 cells/ml). After washing by centrifugation twice at 750 ***g*** for 3 min, the sperm cell culture was continued in m-M199 at 2.5×10^5 cells/ml. At 3 and 4.5 h after exposure to caffeine, the CTC patterns of the sperm cells were examined.

Statistical analysis

Statistical analyses of results from four to six replicate experiments were used for treatment comparisons and carried out by one-way or two-way ANOVA using the JMP 5.0 (SAS Institute, Inc., Cary, NC, USA) program. If the P value was smaller than 0.05 in ANOVA, Tukey–Kramer's HSD test was followed using the same program. All percentage data were subjected to arc-sine transformation before statistical analysis. For showing percentage data in tables and figures, those data were transformed back to the original percentages. All data are expressed as means ± S.E.M. $P < 0.05$ was considered to be statistically significant.

Results

In the present study, we did not observe any differences in penetration rates, survival rates and CTC patterns among replicated trials (ejaculates) and also among boars used ($P > 0.05$).

Effect of further reduction of co-incubation period (experiment 1)

By reducing the period of co-culture with fresh spermatozoa (2.5×10^4 cells/100 μl droplet) from 30 to 5 min, the incidence of monospermic penetration in the mature oocytes examined increased from 1.9 ± 1.2 to 26.5 ± 3.8%, and the mean number of penetrated sperm cells per oocyte decreased from 9.6 ± 1.0 to 2.7 ± 0.2, without any reduction in the penetration rate (Table 1). Interestingly, the percentage of polyspermic oocytes having condensed or decondensed sperm head(s) and at least one male pronucleus was higher in the longer co-culture duration (Table 1; 51.9 ± 0.7% for 5 min and 91.2 ± 4.2% for 30 min).

Effect of reduced co-incubation period on the number of spermatozoa binding to the zona pellucida (experiment 2)

As shown in Fig. 2, the mean number of spermatozoa binding to the surface of zona pellucida was lower after co-culture of gametes (30 oocytes in 100 μl n-M199–caffeine resuspended spermatozoa at 2.5×10^5 sperm/ml) for 5 min than for 8 h.

Effect of the presence of caffeine during co-incubation (experiment 3)

Gametes were co-incubated in m-M199 or n-M199–caffeine (5 mmol/l) for 5 or 30 min and then an additional culture in m-M199 or n-M199–caffeine until 8 h after insemination. The presence of caffeine during the transient co-culture period improved both the rates of sperm penetration and monospermic penetration in total mature oocytes examined, but did not affect the mean number of penetrated sperm cells per zygote (Table 2). Caffeine during the post co-culture period also increased both the incidence of sperm penetration and the number of penetrated sperm cells per zygote. A longer co-culture period

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Table 1 Effect of the duration of co-incubation on sperm penetration of *in vitro*-matured porcine oocytes at 8 h after insemination[a].

	Co-culture period (min)			
	5	10	15	30
No. of oocytes examined	116	103	136	160
No. (%) of mature oocytes	114 (98.7 ± 0.8)	103 (100)	135 (99.4 ± 0.6)	159 (99.5 ± 0.5)
Penetrated (%)[bc]	91.9 ± 4.7	95.8 ± 1.6	99.3 ± 0.7	100
MPN formed (%)[bd]	98.0 ± 1.2	100	99.0 ± 1.0	100
With sperm head (%)[be]	51.9 ± 0.7[f]	67.7 ± 0.1[fg]	76.4 ± 6.0[gh]	91.2 ± 4.2[h]
Monospermy (%)[bc]	26.5 ± 3.8[f]	10.2 ± 2.3[g]	4.0 ± 1.2[h]	1.9 ± 1.2[h]
Sperm/penetrated egg[b]	2.7 ± 0.2[f]	3.9 ± 0.2[fg]	5.0 ± 0.7[g]	9.6 ± 1.0[h]

[a] After co-culture with spermatozoa, oocytes were washed once and transferred to fresh media containing 5 mmol caffeine/l but without sperm cells. The culture was continued until 8 h after insemination.
[b] Data are given as means ± S.E.M. from four and five replicated experiments.
[c] Percentage of oocytes matured.
[d] Percentage of oocytes penetrated.
[e] Percentage of oocytes that formed at least one male pronucleus (MPN). Condensed and decondensed sperm head are contained.
[f–h] Values with different superscripts within rows are significantly different ($P < 0.05$).

of 30 min, as compared with 5 min duration, did not decrease either the incidence of total sperm penetration or monospermic penetration but increased the mean number of spermatozoa that penetrated into an oocyte.

Furthermore, even if oocytes were co-cultured in the absence of caffeine for 5 or 30 min and then cultured in sperm-free n-M199−caffeine until 8 h after insemination, the rates of sperm penetration and monospermic penetration or the mean number of spermatozoa in a penetrated oocyte were similar to those of oocytes exposed to caffeine during both co-culture and the rest culture (post co-culture) period (Table 2). If gametes were not exposed to caffeine during both co-culture and the rest culture duration, penetration rate was very low (3.3 ± 2.7 and 5.1 ± 2.6% in oocytes co-cultured with spermatozoa for 5 and 30 min respectively). The incidence of oocytes containing both condensed or decondensed sperm head(s) and at least one male pronucleus was lower when the oocytes were co-cultured for a brief period and then followed by culture in the absence of caffeine (Table 2).

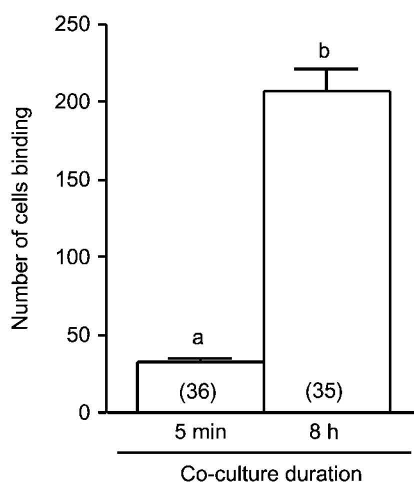

Figure 2 Number (means ± S.E.M.) of spermatozoa binding to the surface of zona pellucida after denuded oocytes were co-cultured with spermatozoa (2.5×10^5 cells/ml) for 5 min or 8 h. Numbers in parentheses indicate the number of oocytes examined in three replicate experiments. Different letters above the bars indicate significant differences ($P < 0.05$).

CTC patterns of spermatozoa after transient exposure to caffeine (experiment 4)

As shown in Fig. 3, survival rate and CTC patterns were affected by both culture periods and treatments. Even transient exposure of spermatozoa to 5 mmol caffeine/l for 5 min reduced the incidence of intact F pattern cells and increased the capacitated B pattern and acrosome-reacted AR pattern cells during culture for 4.5 h as compared with negative controls (not exposed to caffeine). However, the incidence of capacitated F pattern cells was not different from positive controls (exposed to caffeine for the whole duration) but the incidence of acrosome-reacted AR pattern cells was lower.

In vitro *development of oocytes fertilized in conventional or transient IVF systems (experiment 5)*

As shown in Fig. 4, the percentages of cleavage and blastocyst formation were not different among treatment groups.

Effect of preincubation of spermatozoa with adenosine (experiment 6)

As shown in Table 3, regardless of the presence of 10 μmol adenosine/l during preincubation of spermatozoa for 90 min, the percentage of penetrated oocytes was increased when denuded oocytes were co-cultured with spermatozoa in the presence of caffeine. Regardless of the presence of 5 mmol caffeine/l, however, the incidence of monospermic penetration in mature oocytes examined 8 h after insemination was significantly improved when spermatozoa were preincubated in the presence of 10 μmol adenosine/l for 90 min following co-incubation with denuded oocytes. The mean number of sperm cells penetrated into an oocyte was decreased by adenosine during

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Table 2 Effect of the presence of 5 mmol caffeine–benzoate/l during co-incubation and the rest culture until 8 h after insemination on sperm penetration of *in vitro*-matured porcine oocytes.

	Treatments[a]								Significance of effects (*P* value)		
Co-culture duration (min)	5	5	5	5	30	30	30	30	Co-culture duration	Caffeine during co-culture	Caffeine during the rest culture
Concentration of caffeine											
During co-culture	0 mmol/l	0 mmol/l	5 mmol/l	5 mmol/l	0 mmol/l	0 mmol/l	5 mmol/l	5 mmol/l			
During the rest culture	0 mmol/l	5 mmol/l	0 mmol/l	5 mmol/l	0 mmol/l	5 mmol/l	0 mmol/l	5 mmol/l			
No. of oocytes examined	156	156	157	141	167	153	148	158			
No. (%) of mature eggs	142 (91.3 ± 1.1)	153 (98.0 ± 0.8)	148 (94.1 ± 2.1)	140 (99.5 ± 0.5)	151 (90.7 ± 2.1)	151 (98.7 ± 0.8)	144 (97.3 ± 0.7)	157 (99.5 ± 0.5)	0.527	0.031	<0.001
Penetrated (%)[bc]	3.3 ± 2.7	95.3 ± 1.9	84.6 ± 6.9	96.8 ± 3.2	5.1 ± 2.6	96.9 ± 1.9	87.9 ± 5.4	99.5 ± 0.5	0.277	<0.001	<0.001
MPN formed (%)[bd]	75.0 ± 25.0	99.2 ± 0.8	95.4 ± 3.2	98.4 ± 1.0	100	99.2 ± 0.8	87.1 ± 6.0	100	0.167	0.571	0.005
With sperm head (%)[be]	25.0 ± 25.0	51.7 ± 1.6	23.3 ± 3.1	59.3 ± 4.8	11.1 ± 11.1	76.1 ± 8.7	25.0 ± 3.8	86.8 ± 5.7	0.237	0.966	<0.001
Monospermy (%)[bc]	3.3 ± 2.7	24.6 ± 5.8	38.0 ± 4.8	25.4 ± 5.1	4.4 ± 2.3	10.7 ± 5.6	40.6 ± 5.6	3.1 ± 1.3	0.064	<0.001	0.106
Sperm/penetrated egg[b]	1.0	2.8 ± 0.2	1.7 ± 0.1	3.4 ± 0.4	1.1 ± 0.1	5.0 ± 1.4	2.0 ± 0.3	6.9 ± 1.4	0.022	0.108	<0.001

[a] Oocytes were co-cultured with spermatozoa in the absence or presence of 5 mmol caffeine–benzoate/l for 5 or 30 min, transferred to fresh m-M199 containing or not containing 5 mmol caffeine–benzoate/l and then cultured until 8 h after insemination.
[b] Data are given as means ± S.E.M. from five replicated experiments.
[c] Percentage of oocytes matured.
[d] Percentage of oocytes penetrated.
[e] Percentage of oocytes that formed at least one male pronucleus (MPN). Condensed and decondensed sperm head are contained.

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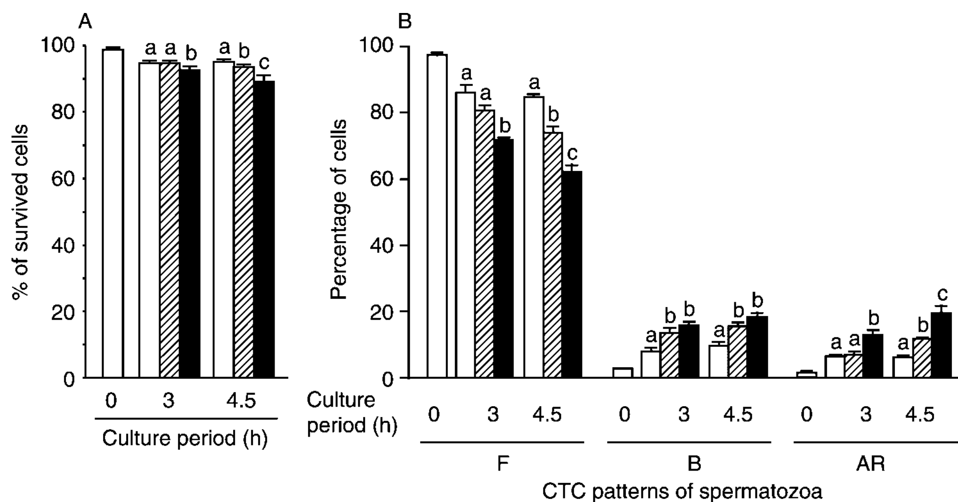

P values	Survival rate	% of F	% of B	% of AR
Culture periods	0.020	<0.001	0.014	<0.001
Treatments	<0.001	<0.001	<0.001	<0.001

Figure 3 Effect of transient exposure to 5 mmol caffeine/l for 5 min on CTC patterns of boar spermatozoa. At 3 and 4.5 h after the start of culture, survival rate (A) and CTC patterns (B) of the spermatozoa (hatched bars) were compared with those of cells exposed (solid bars) or not exposed to caffeine for the whole period (open bars). Different letters above the bars indicate significant differences within the same observation period ($P < 0.05$).

preincubation and increased by caffeine during co-culture. Even if spermatozoa were preincubated in the presence of adenosine for 90 min, the duration of the co-culture period of 5 and 30 min did not affect the penetration rate, incidence of monospermy and mean number of spermatozoa in penetrated oocyte. In this experiment, the incidence of oocytes containing both condensed or decondensed sperm head(s) and at least one male pronucleus was reduced by the presence of adenosine during preincubation (Table 3).

CTC patterns of spermatozoa preincubated with adenosine (experiment 7)

When spermatozoa were preincubated in the absence or presence of 10 μmol adenosine/l for 90 min before transient exposure to 5 mmol caffeine/l for 5 min, both survival rate and CTC patterns were affected by treatment and culture period after the treatment (Fig. 5). The presence of adenosine during preincubation increased the incidence of capacitated B pattern cells and decreased the incidence of acrosome-reacted AR pattern cells even 3 and 4.5 h after transient exposure to caffeine for 5 min.

Discussion

In the present study, we used fresh spermatozoa because cryopreserved boar spermatozoa have been observed to show a capacitation-like reaction (Watson 1995, Green & Watson 2001) and some damage in acrosome and flagella regions (Courtens & Paquignon 1985).

The incidence of polyspermic fertilization in penetrated oocytes has been demonstrated to strongly correlate with the penetration rate (Marchal *et al.* 2002), meaning that there is a possibility that a significant reduction in the polyspermy rate does not always mean an increased number of mature oocytes penetrated by a single sperm cell. In the present study, therefore, to compare the effect of various treatments on monospermic penetration directly, the incidence is shown as a percentage of total mature oocytes examined. Furthermore, we have shown the percentage of oocytes having both at least one male pronucleus and condensed or decondensed sperm head(s).

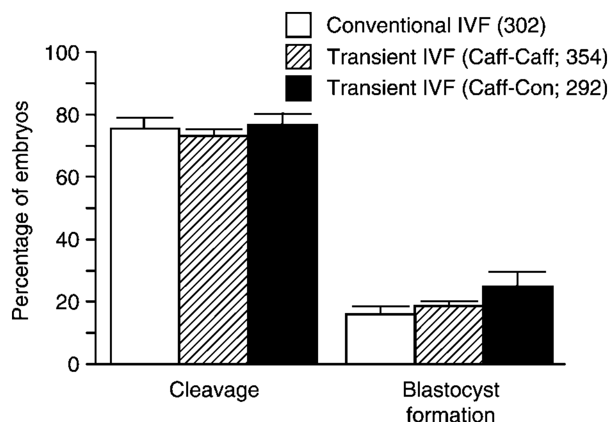

Figure 4 *In vitro* development of oocytes fertilized in a transient IVF system. After transient co-incubation with spermatozoa (2.5×10^5 cells/ml) for 5 min, the oocytes were transferred to m-M199 with (Caff-Caff; hatched bars) or without 5 mmol caffeine/l (Caff-Con; solid bars) and cultured for 8 h. The oocytes were then transferred again to modified NCSU-37 and cultured for 7 days. As a control, oocytes were co-cultured with spermatozoa (2.5×10^5 cells/ml) in a conventional IVF system (m-M199 with 5 mmol caffeine/l) for 8 h (open bars), then transferred to modified NCSU-37 and cultured for 7 days. No significant differences were observed on cleavage or blastocyst formation. Numbers in parentheses indicate the number of oocytes examined in five replicated experiments.

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Table 3 Effect of preincubation of spermatozoa with 10 μmol adenosine/l for 90 min before insemination on sperm penetration of *in vitro*-matured porcine oocytes in a transient co-culture IVF system.

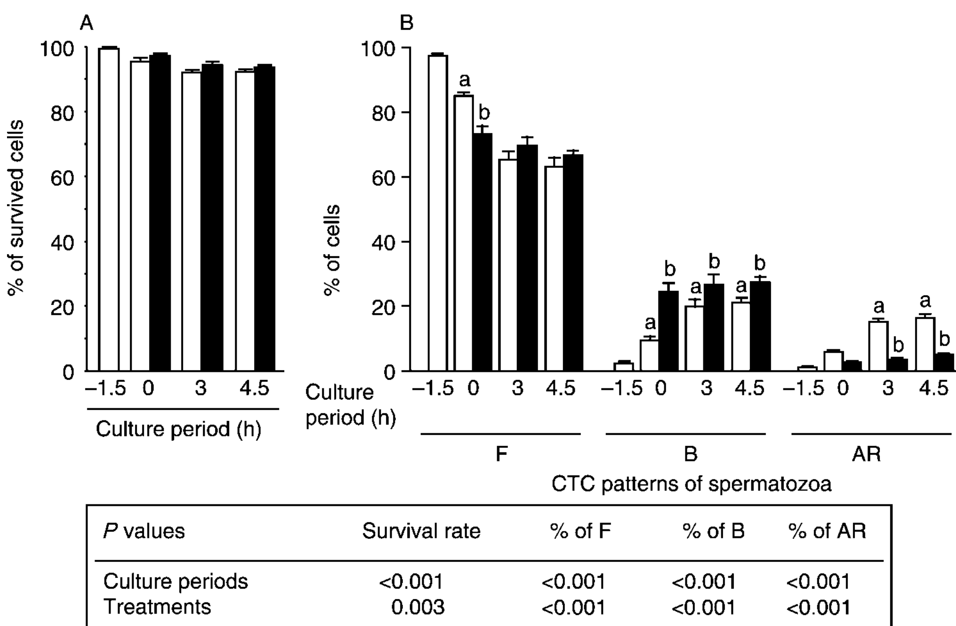
	Treatments[a]					Significance of effects (*P* value)		
Co-culture duration (min)	30	30	30	30	5			
Presence of adenosine during preincubation	0 μmol/l	0 μmol/l	10 μmol/l	10 μmol/l	10 μmol/l	Duration of co-culture	Adenosine during preincubation	Caffeine during co-culture
Presence of caffeine during co-culture	0 mmol/l	5 mmol/l	0 mmol/l	5 mmol/l	5 mmol/l			
No. of occytes examined	164	151	156	165	164			
No. (%) of mature eggs	145 (89.8 ± 1.6)	147 (97.7 ± 1.4)	144 (92.4 ± 1.5)	151 (91.5 ± 1.4)	155 (94.5 ± 1.5)	0.472	0.542	0.040
Penetrated (%)[bc]	34.1 ± 6.8	88.6 ± 5.9	52.6 ± 3.6	81.1 ± 2.3	82.6 ± 2.1	0.376	0.552	<0.001
MPN formed[bd]	91.1 ± 4.1	96.5 ± 1.8	88.3 ± 3.2	93.6 ± 1.8	95.9 ± 1.5	0.318	0.601	0.029
With sperm head (%)[be]	31.8 ± 4.9	50.4 ± 12.1	23.8 ± 1.1	24.5 ± 3.9	25.7 ± 9.6	0.493	0.022	0.404
Monospermy (%)[bc]	29.0 ± 4.3	23.6 ± 5.5	42.9 ± 4.1	47.8 ± 3.7	48.5 ± 1.5	0.092	<0.001	0.821
Sperm/penetrated egg[b]	1.2 ± 0.1	3.1 ± 0.8	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	0.633	0.008	0.002

[a] After preincubation of spermatozoa for 90 min, oocytes were co-cultured with the spermatozoa for 5 or 30 min, transferred to fresh m-M199 and then cultured until 8 h after insemination.
[b] Data are given as means ± S.E.M. from five replicated experiments.
[c] Percentage of oocytes matured.
[d] Percentage of oocytes penetrated.
[e] Percentage of oocytes that formed at least one male pronucleus (MPN). Condensed and decondensed sperm head are contained.

Since various morphological types of sperm nucleus can be observed in polyspermic oocytes, a difference in the percentage may reflect a change of the width of the window for sperm penetration.

In the current study, spermatozoa bound to the oocytes during co-culture for 5 or 30 min even in the absence of caffeine, because high penetration rates were obtained when the oocytes were followed by culture in the presence of caffeine (Table 2). However, the spermatozoa could not achieve penetration if the oocytes were continued to be cultured in a caffeine-free medium. Uncapacitated acrosome-intact spermatozoa can bind to the zona pellucida (Peterson *et al.* 1984), and zona pellucida can initiate acrosome reaction of boar spermatozoa *in vitro* (Berger *et al.* 1989, Melendrez *et al.* 1994). In the present result of the CTC assay, 96.7 ± 0.5% of spermatozoa were intact (uncapacitated acrosome-intact) cells at insemination. The current results suggest that uncapacitated acrosome-intact spermatozoa bound to the zona pellucida can penetrate if the cells are stimulated by inducers for capacitation/acrosome reaction, such as caffeine.

Polyspermic penetration into porcine oocytes *in vitro* is more likely due to an incomplete or slow zona reaction or simultaneous sperm penetration rather than an abnormal cortical reaction (Wang *et al.* 1999). One strategy to overcome this abnormality or characteristic in porcine oocytes may be by trying to induce a complete and quick zona reaction in response to cortical exocytosis, and another



P values	Survival rate	% of F	% of B	% of AR
Culture periods	<0.001	<0.001	<0.001	<0.001
Treatments	0.003	<0.001	<0.001	<0.001

Figure 5 Effect of preincubation with 10 μmol adenosine/l on CTC patterns of boar spermatozoa after transient exposure to 5 mmol caffeine/l for 5 min. At 3 and 4.5 h after the transient exposure to caffeine, survival rate (A) and CTC patterns (B) of the spermatozoa preincubated with (solid bars) or without adenosine for 90 min (open bars) were examined. Different letters above the bars indicate significant differences within the same observation period ($P < 0.05$).

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strategy should be to reduce the number of spermatozoa that achieve zona penetration during the relatively wide window before the zona reaction by regulating the sperm–zona pellucida binding. In a majority of porcine IVF systems (Cheng *et al.* 1986, Yoshida 1987, Nagai *et al.* 1988, Mattioli *et al.* 1989), gametes have been co-cultured in droplets of medium containing caffeine for several hours or overnight. In a conventional IVF system, both the incidence of oocytes penetrated and the percentage of polyspermy in the penetrated oocytes also appear to increase in the presence of caffeine with the duration of the co-culture and sperm concentration (Funahashi & Nagai 2001). In the present study, we first reduced the duration of the co-incubation of oocytes with fresh spermatozoa in the presence of caffeine to confirm a concept that was demonstrated previously with frozen–thawed spermatozoa. We demonstrated here that the number of spermatozoa binding to the zona pellucida and the incidence of polyspermic penetration could be decreased by shortening the duration of co-incubation of gametes. These results were consistent with previous reports with frozen–thawed spermatozoa (Gil *et al.* 2004), demonstrating that shortening the co-culture duration to 10–30 min decreased both the number of spermatozoa bound to the zona pellucida and the incidence of polyspermy. Here we have indicated that fresh spermatozoa reduced the chance of sperm binding to the oocytes and consequently achieved a lower incidence of polyspermic penetration after shorter co-culture of gametes. However, our result showed that even fresh boar spermatozoa bound to the oocytes within 5 min after insemination contributed to the polyspermic fertilization in about 65% of the inseminated oocytes if an additional culture in the presence of caffeine followed. In these conditions, we also found that the incidence of IVF oocytes containing both at least one male pronucleus and condensed or decondensed sperm head(s) increased in longer co-culture periods. In the presence of caffeine even after a co-culture period, the window for sperm penetration may have become wider when a larger number of spermatozoa bound to the zona pellucida during co-culture.

In the current study, we examined the effect of caffeine during a transient co-culture and the rest culture period on sperm penetration. Caffeine has been used to induce sperm capacitation in a majority of current porcine IVF systems (Cheng *et al.* 1986, Yoshida 1987, Nagai *et al.* 1988, Mattioli *et al.* 1989). We have shown here that a transient exposure to 5 mmol caffeine/l for 5 min is enough to induce sperm capacitation and penetration. We also demonstrated that if the oocytes were continued in culture in the absence of caffeine, the number of sperm cells penetrated within the window until the completion of the zona reaction following the first sperm penetration could be reduced because of a limited stimulation of acrosome reaction. Furthermore, the window of sperm penetration itself appears to be narrowed because the incidence of oocytes having both male pronucleus and condensed or decondensed sperm head(s) significantly decreased in this condition. If the transient co-culture was followed by an additional culture in the presence of caffeine, capacitation/acrosome reaction was stimulated and the incidence and number of spermatozoa penetrated significantly increased. These results suggest that not all spermatozoa bound to the oocytes but only a limited number of the cells seem to stimulate penetrability by caffeine during the transient co-incubation. Therefore, although there is a high correlation between the incidence of polyspermy in penetrated oocytes and the ratio of spermatozoa/oocyte penetrated in the fertilization medium (Rath 1992), we propose here that a brief period of exposure of the gametes to caffeine is also an important factor in preventing multiple penetration. The current new IVF method, a transient co-incubation in the presence of caffeine followed by culture of the oocytes in caffeine-free medium, will be useful to further increase the incidence of normal fertilization in porcine oocytes *in vitro* by controlling the sperm–zona binding and the penetrable window of spermatozoa.

However, a transient exposure of gametes to caffeine during a brief co-culture period did not increase the efficiency of cleavage and blastocyst formation. This result was consistent with a recent publication (Gil *et al.* 2004). It has also been demonstrated that polyspermic porcine IVM–IVF embryos containing multiple pronuclei can develop *in vitro* to the blastocyst stage at the same percentage as monospermic IVM–IVF embryos (Han *et al.* 1999).

It has recently been demonstrated that both adenosine and FPP can regulate the function of freshly ejaculated and frozen–thawed boar spermatozoa (Funahashi *et al.* 2000*b*). Replacement of caffeine with adenosine or FPP in the fertilization medium in a conventional IVF system improved monospermic penetration by frozen–thawed boar spermatozoa (Funahashi *et al.* 2000*c*, Funahashi & Nagai 2001). In the present study using fresh spermatozoa, we showed that preincubation of boar spermatozoa in the presence of adenosine for 90 min significantly increased the incidence of oocytes penetrated by a single spermatozoon and reduced the incidence of oocytes containing both male pronucleus and condensed or decondensed sperm head(s), regardless of the duration of the co-culture period within 30 min and of the presence of caffeine during co-incubation. These results could be due to the phenomenon that acrosome reaction of adenosine-treated cells is prevented even after a transient exposure to caffeine. These results demonstrated that a combination of preincubation of fresh spermatozoa with adenosine and a transient co-incubation IVF system is effective at narrowing the window of sperm penetration and increasing the incidence of oocytes penetrated by a single spermatozoon. Adenosine stimulates and prevents the capacitation and spontaneous acrosome reaction of boar spermatozoa respectively, via the adenylyl cyclase/cAMP pathway (Funahashi *et al.* 2000*b*). In the presence of adenosine, the timing of *in vitro* penetration of frozen–thawed spermatozoa appears to be slower than that in the presence of

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caffeine (Funahashi & Nagai 2001). In this combined IVF system, therefore, a limited number of spermatozoa capacitated by preincubation with adenosine are induced in the zona-initiated acrosome reaction (Berger *et al.* 1989, Melendrez *et al.* 1994) and may penetrate the zona pellucida in a scattered manner.

In the present study, we focused only on an IVF system permitting simultaneous sperm penetration. Although treatments affecting sperm function, such as pretreatment with adenosine before transient exposure to caffeine, reduced polyspermic penetration rate and the width of the window for sperm penetration, the incidence was still unacceptable for commercial application. Ultrastructural observations of the zona pellucida of porcine oocytes (Funahashi *et al.* 2000*a*, 2001) have found morphological differences in the zona pellucida of oocytes matured *in vitro* and *in vivo* and these authors hypothesized that the differences due to a failure in final maturation may result in incomplete or delayed zona reaction. Polyspermy rate appears to be affected by the donor of oocytes (Marchal *et al.* 2001) and is still higher for *in vitro* matured oocytes than for ovulated oocytes (Wang *et al.* 1998). Improvement of the quality of porcine IVM oocytes and use of the oocytes in the current IVF system may help to overcome polyspermy. Furthermore, recent studies have demonstrated that exposure to porcine oviduct-specific glycoproteins before and during IVF increases the penetrability (Romar *et al.* 2003) and reduces the incidence of polyspermy in pig oocytes (Romar *et al.* 2001), and the number of bound sperm (Kouba *et al.* 2000, McCauley *et al.* 2003). Exogenous hyaluronan, which has been detected in porcine oviduct fluid (Tienthai *et al.* 2000), has also been demonstrated to decrease polyspermy during conventional porcine IVF (Suzuki *et al.* 2000). Sperm treatments before insemination (Matas *et al.* 2003), IVF media (Coy *et al.* 2002) and IVF cambers (Funahashi & Nagai 2000, Li *et al.* 2003, Wheeler *et al.* 2004) also have been known to affect the incidence of polyspermic penetration in porcine IVF systems. Further research should be conducted into these techniques in the current transient co-incubation IVF system.

In the present study, a mixture of caffeine and sodium benzoate at equal weight was used as a caffeine reagent. Due to the presence of sodium benzoate, adding 5 mmol caffeine/l may increase the osmolarity of the medium. Therefore, the current IVF results may contain an effect of the exposure of the gamete, not only to caffeine, but also to different osmolarities for various durations. Furthermore, since we focused on examining the effects of the duration of exposure of the gamete to caffeine, we used a concentration of caffeine at 5 mmol/l in the current study. Adjustment of the kind and concentration of caffeine should be carried out in further experiments to obtain a higher incidence of normal penetration.

In conclusion, our present study has demonstrated that a transient co-incubation of oocytes with spermatozoa in the presence of caffeine for 5 or 30 min followed by an additional culture in the absence of caffeine reduces the incidence of oocytes penetrated by multiple spermatozoa, suggesting that a transient duration of exposure of gametes to caffeine is an important factor in preventing polyspermic penetration into porcine oocytes. Furthermore, preincubation of spermatozoa with adenosine before a transient co-incubation narrows the window for sperm penetration and improves the incidence of monospermic fertilization, regardless of the presence of caffeine during the co-incubation period.

Acknowledgements

We thank Okayama Prefectural Center for Animal Husbandry and Research for supplying fresh boar semen. This work was supported in part by grants to H F from the Ito Foundation and Grant-in-Aid for Scientific Research (No. 16580230) of the Japan Society for the Promotion of Science and to R R from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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Received 29 April 2004
First decision 3 June 2004
Revised manuscript received 13 August 2004
Accepted 16 August 2004

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