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Effects of sperm concentrations and culture media on fertilization and development of *in vitro* matured pig oocytes

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Summary

This study was carried out to investigate the effects of sperm concentrations and culture media on fertilization and development of *in vitro* matured pig oocytes. The concentrations of frozen-thawed sperm were 0.2×10^7, 2×10^7, 20×10^7 and 200×10^7/ml, respectively. Culture media were NCSU-23, HEPES-buffered (25 mM) NCSU-23, PZM-3 and PZM-4, respectively. Increasing the sperm concentration from 0.2×10^7 to 2×10^7/ml, significantly increased the penetration rate. Also, increasing the sperm concentration from 20×10^7 to 200×10^7/ml increased the penetration rate from 62.1% to 69.9%, respectively, with no differences between these two concentrations. A similar pattern was observed for polyspermic penetration and male pronucleus formation. The mean number of sperm per oocyte significantly increased in the 20×10^7/ml and again in the 200×10^7/ml sperm concentrations. The percentage of blastocysts from cleaved oocytes at the 2×10^7/ml sperm concentration was significantly higher than that at the 0.2×10^7, 20×10^7 and 200×10^7/ml sperm concentrations. The percentage of blastocysts from cleaved oocytes and the cell numbers per blastocyst were significantly higher in the HEPES-buffered NCSU-23 culture medium than in the NCSU-23, PZM-3 and PZM-4 culture media under a gas atmosphere of 5% CO_2 in air.

Keywords: Blastocyst, Culture medium, *In vitro* fertilization, Pig oocyte, Sperm concentration

Introduction

Successful *in vitro* fertilization of pig oocytes using freshly ejaculated spermatozoa has been reported (Cheng, 1985; Yoshida, 1987). However, this procedure is subject to variability caused both by variability amongst boars and by subtle differences in the condition of the semen that is used. To obtain repeatable *in vitro* fertilization results with a high success rate, use of standardized frozen-thawed spermatozoa would be a considerable advantage.

When *in vitro* fertilization was carried out with frozen-thawed sperm concentrations of 5×10^5/ml and 1×10^6/ml for 12 h in mTBM medium, penetration rate reached 84% and 86%, respectively. The polyspermy rate was 57% and 64%, respectively; the difference was not statistically significant. However, the mean number of sperm per oocyte was significantly increased when sperm concentration was increased above 1×10^6/ml (Abeydeera & Day, 1997). Similar higher penetration rates were also observed by Wang *et al*. (1991) in their study using frozen-thawed ejaculated boar semen. However, their study required a very much higher sperm concentration (25×10^6/ml). Hyperactivation and the acrosome reaction occurred after capacitation and were crucial for successful fertilization (Yanagimachi, 1994). Thus, when culture conditions did not produce efficient capacitation, the relatively high concentration of spermatozoa needed for insemination was thought to be a major factor contributing to the high incidence of polyspermic penetrations, leading to a low rate of blastocyst formation (Funahashi & Day, 1997).

Pig embryos can develop from the zygote to the blastocyst stage *in vitro*. Several media, such as modified Whitten medium (Beckmann & Day, 1993), North Carolina State University (NCSU)-23 medium (Petters & Wells, 1993), Beltsville embryo culture

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medium (BECM)-3 (Dobrinsky *et al*., 1996), porcine zygote medium (PZM)-3 and PZM-4, allowed the successful culture of embryos to the blastocyst stage. In particular, NCSU-23 was the most successful medium compared with other culture media for the culture of pig embryos derived *in vivo* and *in vitro* to the blastocyst stage (Petters & Wells, 1993; Macháty *et al*., 1998; Long *et al*., 1999).

Recently, Yoshioka *et al*. (2002) reported that culture of zygotes in PZM containing 3 mg/ml of bovine serum albumin (BSA), i.e. PZM-3, produced better results in terms of the proportion of day 6 blastocysts, day 8 hatching rate, and numbers of inner cell mass (ICM) cells and total cells in day 8 embryos than did NCSU-23 medium. The ICM and total cell numbers in day 6 embryos cultured in PZM-3 or in PZM-3 in which BSA was replaced with 3 mg/ml of polyvinyl alcohol (PZM-4) were also greater than those in NCSU-23 but less than those in embryos developed *in vivo*. Iwasaki *et al*. (1999) reported that when HEPES buffer was added, the pH was stabilized. When the pH of the medium was unstable, embryos could not be left outside the CO_2 incubator.

The aim of this study was to investigate the effects of different concentrations of frozen-thawed sperm and to compare the effects of NCSU-23, HEPES-buffered (25 mM) NCSU-23, PZM-3 and PZM-4 media on the fertilization and development of *in vitro* matured pig oocytes.

Materials and methods

Semen collection

Semen was collected from three Duroc boars twice weekly. The filtered sperm-rich fraction was collected by the gloved-hand technique into a 250 ml insulated vacuum bottle. The sperm-rich fractions of ejaculates with greater than 85% motile sperm and NAR acrosome were used.

Frozen semen processing

The sperm-rich fraction (30–60 ml) of ejaculates was collected into an insulated vacuum bottle. Semen was slowly cooled to room temperature (20–23 °C) by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800 *g*, and the supernatant solution poured off. One volume of concentrated sperm was resuspended with 1 volume of lactose, egg yolk and *N*-acetyl-D-glucosamine (LEN) diluent (the first diluent to provide 1×10^9 sperm/ml) at room temperature (Yi *et al*., 2002).

Semen was cooled in a refrigerator to 5 °C over a 2 h period and 1 volume a LEN + 4% glycerol diluent (the second diluent) was added to 1 volume of cooled semen. Straws (Minitub, Landshut, Germany) were immediately filled with 5 ml of semen and steel or glass balls were used to seal the ends of the straws. The air bubble was adjusted to the centre of each straw and the straws placed horizontally on an aluminium rack and set into a tank containing liquid nitrogen (LN). The straws were situated 5 cm above the LN, and kept at that level for 20 min before being transferred into LN storage.

Oocyte collection and *in vitro* maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl solution containing 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate and 0.1% BSA at 30–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) using an 18 gauge needle fixed to a 10 ml disposable syringe. COCs were washed three times in mTLP-PVA and two times with a maturation medium. Thirty to forty COCs were transferred to 500 μl of the same medium that had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) and equilibrated at 38.5 °C, 5% CO_2 in air. The medium used for oocyte maturation was tissue culture medium (TCM)-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg/ml insulin, 2 μg/ml vitamin B_{12}, 25 mM HEPES, 10 μg/ml bovine apotransferrin, 150 μM cysteamine, 10 IU/ml PMSG, 10 IU/ml hCG, 10 ng/ml EGF, 0.4% BSA, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 10% pFF. After about 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5 °C, in 5% CO_2 in air.

In vitro fertilization and culture of oocytes

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in mTLP-PVA and washed two times with mTBM fertilization medium. Thereafter, 30–40 oocytes were transferred into each well of a 4-well multidish containing 500 μl mTBM fertilization medium that had been covered with mineral oil and equilibrated at 38.5 °C, in 5% CO_2 in air.

For IVF, one frozen 5 ml straw was thawed at 52 °C in 40 s and was diluted with 20 ml Beltsville thawing solution (BTS) at room temperature. Two millilitres of the diluted sperm was then added to 8 ml of mTLP-PVA and centrifuged two times for 5 min at 800 *g*. Washed sperm was resuspended with mTBM, and oocytes were inseminated with 0.2×10^7, 2×10^7, 20×10^7 and 200×10^7/ml sperm concentrations, respectively, to investigate the effects of sperm concentration, and with 2×10^7/ml sperm concentration to compare the

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Table 1 Effects of sperm concentration on fertilization parameters of pig oocytes matured *in vitro*

Sperm concentration ($\times 10^7$/ml)	No. of oocytes inseminated	% of oocytes penetrated[1]	% of polyspermic oocytes[1]	% of oocytes with male pronucleus[1]	Mean no. of sperm per penetrated oocyte[1]
0.2	111	34.9 ± 3.1^{c}	1.1 ± 1.1^{c}	23.8 ± 3.3^{c}	0.4 ± 0.2^{c}
2	107	53.9 ± 3.3^{b}	4.1 ± 2.2^{bc}	48.0 ± 3.0^{b}	1.5 ± 0.2^{c}
20	108	62.1 ± 2.0^{ab}	13.1 ± 3.1^{b}	54.7 ± 1.7^{ab}	4.6 ± 0.6^{b}
200	104	69.9 ± 2.5^{a}	27.5 ± 4.9^{a}	60.9 ± 1.9^{a}	7.6 ± 1.0^{a}

[1] Mean ± SE. Experiments were repeated five times.
[abc] Values in the same column with different superscripts differ significantly, $p < 0.05$.

effects of culture media. At 6 h after IVF, oocytes were transferred into 500 µl HEPES-buffered (25 mM) NCSU-23 culture medium to investigate the effects of sperm concentrations and into 500 µl NCSU-23, HEPES-buffered NCSU-23, PZM-3 and PZM-4 culture media to compare the effects of culture media.

Examination of oocytes

At 12 h and 48 h after insemination, oocytes were fixed for 48 h in 25% acetic acid (v:v) in ethanol at room temperature, and stained with 1% (w:v) orcein in 45% (v:v) acetic acid to examine metaphase II, sperm penetration, polyspermic oocytes, male pronucleus and cleaved oocytes under a phase-contrast microscope at × 400 magnification. Blastocysts on day 6 were stained with Hoechst 33342 and the number of nuclei counted under a fluorescence microscope (Olympus, Japan).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS Institute, 1996) in a completely randomized design. Duncan's multiple range test was used to compare mean values of individual treatment when the F value was significant ($p < 0.05$).

Results

Effects of sperm concentrations on fertilization parameters of pig oocytes matured *in vitro*

As shown in Table 1, increasing the sperm concentration from 0.2×10^7 to 2×10^7 significantly increased the penetration rate from 34.9% to 53.9%, respectively. Also, increasing the sperm concentration from 20×10^7 to 200×10^7/ml increased the penetration rate from 62.1% to 69.9%, respectively, though this difference was not statistically significant.

Table 2 Effects of sperm concentration on the developmental ability of pig embryos[1]

Sperm concentration ($\times 10^7$/ml)	No. of embryos cultured[2]	% of cleaved oocytes[3]	% blastocysts from cleaved oocytes[3]
0.2	107	63.9 ± 2.9^{b}	6.8 ± 1.5^{b}
2	107	72.5 ± 3.2^{a}	17.4 ± 2.8^{a}
20	103	73.1 ± 2.6^{a}	10.7 ± 2.0^{b}
200	102	73.5 ± 2.0^{a}	8.8 ± 1.9^{b}

[1] Mean ± SE. Experiments were repeated five times.
[2] Cultured oocytes were selected after *in vitro* fertilization (IVF). The oocytes were cultured in HEPES-buffered NCSU-23 culture medium.
[3] Rates of cleavage and blastocyst were examined at 48 and 144 h after IVF, respectively.
[ab] Values in the same column with different superscripts differ significantly, $p < 0.05$.

A similar pattern was observed for polyspermic penetration and male pronucleus formation. The mean number of sperm per oocyte significantly increased in the 20×10^7/ml and again in the 200×10^7/ml sperm concentration.

Effects of sperm concentration on developmental ability of pig embryos

As shown in Table 2, the rates of cleaved oocytes with the 0.2×10^7/ml sperm concentration was significantly lower than those at the 2×10^7, 20×10^7 and 200×10^7/ml sperm concentrations. However, the percentage of blastocysts from cleaved oocytes at the 2×10^7/ml sperm concentration was significantly higher than those at the 0.2×10^7, 20×10^7 and 200×10^7/ml sperm concentrations.

In vitro culture of pig embryos

As shown in Table 3, the percentage of cleaved oocytes was significantly higher in the NCSU-23 and HEPES-buffered NCSU-23 culture media than in the PZM-3 and PZM-4 culture media. The percentage of

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Table 3 Effects of culture medium on the developmental ability of pig embryos[1]

Culture medium	No. of embryos cultured[2]	% of cleaved oocytes[3]	% blastocysts from cleaved oocytes[2]	Cell no. per blastocyst
NCSU-23	145	74.7 ± 2.6^a	12.7 ± 1.8^b	26.7 ± 2.4^b
HEPES-buffered NCSU-23	148	75.6 ± 2.7^a	18.0 ± 3.0^a	34.3 ± 3.7^a
PZM-3	150	68.7 ± 1.7^b	10.3 ± 1.9^b	25.5 ± 1.9^b
PZM-4	151	67.1 ± 1.9^b	9.5 ± 1.7^b	23.2 ± 1.3^b

[1] Mean ± SE. Experiments were repeated four times.
[2] Cultured oocytes were selected after *in vitro* fertilization (IVF). The oocytes were inseminated with 2×10^7/ml sperm concentration in mTBM fertilization medium.
[3] Rates of cleavage and blastocysts were examined at 48 and 144 h after IVF, respectively.
[ab] Values in the same column with different superscripts differ significantly, $p < 0.05$.

blastocysts from cleaved oocytes and the cell numbers per blastocyst were significantly higher in the HEPES-buffered NCSU-23 culture medium than in the NCSU-23, PZM-3 and PZM-4 culture media.

Discussion

An optimal number of fully capacitated spermatozoa at the site of fertilization is extremely important for reducing the incidence of polyspermy in pigs (Rath, 1992; Coy *et al*., 1993; Xu *et al*., 1996) as it not only maintains a high proportion of oocytes being fertilized but also avoids a high rate of polyspermic penetration.

Wang *et al*. (1991) reported that few oocytes were penetrated at sperm concentrations of $1–6 \times 10^6$sperm/ml, but that high penetration rates (85–89%) and an increased incidence of polyspermy were obtained with more than 25×10^6 sperm/ml on BF_5 (Pursel & Johnson, 1975) frozen-thawed sperm. Also 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. When *in vitro* fertilization was carried out with frozen-thawed sperm concentrations of 5×10^5/ml and 1×10^6/ml for 12 h fertilization in mTBM, penetration rates reached 84% and 86%, respectively. The polyspermy rate was 57% and 64%, respectively, the difference not being statistically significant. However, the mean number of sperm per oocyte was increased when sperm concentration was increased to 1×10^6/ml (Abeydeera & Day, 1997). According to Hunter & Nichol (1988), the incidence and degree of polyspermy was an indication of the number of capacitated spermatozoa present in the immediate vicinity of the ovulated oocytes *in vivo*.

In the present study, a similar pattern was observed for penetration rate, polyspermic penetration and male pronucleus formation. Also, the mean number of sperm per oocyte significantly increased in the 20×10^7/ml and again in the 200×10^7/ml sperm concentration. The rate of cleaved oocytes at the 0.2×10^7/ml sperm concentration was significantly lower than those at 2×10^7, 20×10^7 and 200×10^7/ml.

Macháty *et al*. (1998) reported that culture of pig embryos in NCSU-23 resulted in an increased number of trophectoderm cells and total cells in day 6 blastocysts that developed in 5% CO_2 in air (20% O_2) compared with those developed in 5% O_2. Yoshioka *et al*. (2002) reported that no significant effect of oxygen tension on embryo development in the culture with NCSU-23 was observed. However, culture of porcine zygotes at 5% O_2 produced better results in terms of the proportion of day 6 blastocysts, day 8 hatching rate, and ICM and total cell numbers of day 8 embryos than did culture in 20% O_2 when PZM-3 and PZM-4 were used.

In the present study, the percentage of blastocysts from cleaved oocytes and the cell numbers per blastocyst were significantly higher in the HEPES-buffered NCSU-23 culture medium than in NCSU-23, PZM-3 and PZM-4 media. These results indicate that PZM appears to provide appropriate conditions for culture of porcine zygotes under low-oxygen conditions rather than under a gas atmosphere of 5% CO_2 in air. Also, addition of HEPES buffer to NCSU-23 culture medium seems to provides a better environment for the development of porcine zygotes. Iwasaki *et al*. (1999) reported that when HEPES buffer was added, the pH was stabilized.

In conclusion, when *in vitro* fertilization was carried out with frozen-thawed sperm, the optimum concentration in mTBM fertilization medium was 2×10^7/ml. The HEPES-buffered NCSU-23 culture medium was the most successful for the culture of pig embryos compared with NCSU-23, PZM-3 and PZM-4 culture media under a gas atmosphere of 5% CO_2 in air.

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