

Liquid Boar Sperm Quality during Storage and *In vitro* Fertilization and Culture of Pig Oocytes

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ABSTRACT : The percentages of sperm motility and normal acrosome on the liquid boar semen diluted and preserved at 4°C with lactose hydrate, egg yolk and N-acetyl-D-glucosamine (LEN) diluent were significant differences according to preservation day and incubation time, respectively. The sperm motility steadily declined from 96.9% at 0.5 h incubation to 78.8% at 6 h incubation at 1 day of preservation. However, the sperm motility rapidly declined after 4 day of preservation during incubation. The normal acrosome steadily declined from 93.3% at 0.5 h incubation to 73.8% at 6 h incubation at 1 day of preservation. However, the normal acrosome rapidly declined after 3 day of preservation during incubation. The rates of sperm penetration and polyspermy were higher in 5 and 10×10⁶ sperm/ml than in 0.2 and 1×10⁶ sperm/ml. Mean numbers of sperm in penetrated oocyte were highest in 10×10⁶ sperm/ml compared with other sperm concentrations. The rates of blastocysts from the cleaved oocytes (2-4 cell stage) were highest in 1×10⁶ sperm/ml compared with other sperm concentrations. In conclusion, we found out that liquid boar sperm stored at 4°C could be used for *in vitro* fertilization of pig oocytes matured *in vitro*. Also, we recommend 1×10⁶ sperm/ml concentration for *in vitro* fertilization of pig oocytes. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 10 : 1369-1373)

Key Words : *In vitro* Fertilization, Pig Oocyte, Liquid Boar Sperm

INTRODUCTION

In vitro evaluation of sperm quality is important for research on sperm preservation and *in vitro* fertilization (IVF) of pig oocytes. Recently, Sellés et al. (2003) reported that the IVF system was a good tool to evaluate the quality of frozen-thawed boar semen previous to its commercial way and a good way to assay new sperm freezing procedures, as it was the more precise evaluating method in estimating the potential fertilizing ability.

Successful IVF could be induced effectively when both fresh boar ejaculate (Nagai et al., 1984; Hamano and Toyoda, 1986) and epididymal sperm (Nagai et al., 1984) were preincubated at a high density in a suitable medium before IVF. These reports suggested that preincubation was important for sperm capacitation. However, frozen and thawed boar ejaculated sperm were capable of IVF not only after preincubation (Nagai et al., 1988), but also without preincubation (Wang et al., 1991). With the development of rapid transportation, and satisfactory preservation of liquid boar semen at about 18°C (Weitze, 1991; Johnson, 1998) or 5°C (Park et al., 1992), artificial insemination of pig has

increased rapidly. But IVF of *in vitro* matured pig oocytes using liquid boar sperm stored at 4°C was not reported.

Therefore, this study was carried out to evaluate liquid boar sperm quality during storage, and investigate IVF and culture of pig oocytes inseminated by liquid boar sperm preserved at 4°C.

MATERIALS AND METHODS

Semen collection and liquid semen processing

Semen was collected from one adult Yorkshire boar twice weekly. Boar was housed at Division of Animal Science & Resources, Chungnam National University. The sperm-rich fraction (30 to 60 ml) of ejaculate was collected into an insulated vacuum bottle. The sperm-rich fractions of ejaculates with greater than 85% motile sperm and NAR acrosome were used.

Semen was slowly cooled to room temperature (20 to 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 supernatant solution was poured off. The concentrated sperm was resuspended with 5 ml of LEN (11.0 g lactose hydrate, 20.0 ml egg yolk, 0.05 g N-acetyl-D-glucosamine and 100.0 ml distilled water) diluent to provide 1.0×10⁹ sperm/ml at room temperature. The resuspended semen was cooled in a refrigerator to 4°C and preserved for 5 days.

Sperm evaluation

For sperm evaluation preserved at 4°C, 0.5 ml of the

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preserved semen were added to a test tube containing 19.5 ml of BTS (Beltsville thawing solution). One ml aliquot of the diluted semen was then added to 1 ml of 1% glutaraldehyde in BTS for the 0 h acrosome morphology evaluation and 19 ml were incubated at 37°C. After 0.5 h incubation, 10 µl aliquot were transferred onto glass slides and 18×18 mm cover-slips were applied. The percentage of motile sperm was estimated at 37°C by light microscope at 250×.

The acrosome morphology of 100 sperm per sample was evaluated by phase contrast microscopy at 100×. Acrosomes were differentially categorized into four morphological classes: normal apical ridge (NAR), damaged apical ridge (DAR), missing apical ridge (MAR) and loose acrosomal cap (LAC) as described by Pursel et al. (1972). All samples were coded and the identify of the treatment was unknown during evaluation.

Oocyte collection and *in vitro* maturation

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% NaCl solution containing 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulfate at 30-35°C. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3 to 6 mm in diameter) using a 18 gauge needle fixed to a 10 ml disposable syringe. COCs were washed three times in mTLP-PVA and were washed twice with a maturation medium. 30-40 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% CO₂ 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 B₁₂, 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. About 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% CO₂ in air.

In vitro fertilization and culture of oocytes

After the completion of culture of oocytes for *in vitro* maturation, cumulus cells were removed with 0.1% hyaluronidase in mTLP-PVA and washed twice with mTBM fertilization medium. Thereafter, 30-40 oocytes were transferred into each well of a 4 well multidish containing 500 µl mTBM that had been covered with mineral oil and equilibrated at 38.5°C, 5% CO₂ in air. The dishes were kept in a CO₂ incubator until spermatozoa were added for insemination.

For IVF, 0.5 ml liquid semen showing more than 80%

sperm motility and normal acrosome at 0.5 and 0 h incubation time, respectively, was diluted with 19.5 ml Beltsville thawing solution (BTS) at 4°C. Sperm were washed twice in mTLP-PVA and then resuspended with mTBM at room temperature. Oocytes and diluted sperm were coincubated for 6 h in 500 µl mTBM (Yi et al., 2004). At 6 h after IVF, oocytes were transferred into 500 µl NCSU-23 culture medium containing 25 mM Hepes and 0.4% BSA for further culture of 6, 48 and 144 h.

Examination of oocytes

At 12 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 sperm penetration, polyspermic oocyte, male pronucleus, number of penetrated sperm in oocyte and cleaved oocytes under a phase-contrast microscope at ×400 magnification. Blastocysts at 144 h were stained with Hoechst 33342 and were counted nucleus number under fluorescent microscope (Olympus, Japan).

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

Experimental design

Experiment 1 was conducted to investigate the percentage of sperm motility and normal acrosome according to preservation day from 1 to 5 day and incubation time, respectively. Samples were incubated for 0.5, 2, 3, 4, 5 and 6 h at 37°C per day.

Experiment 2 was carried out to investigate the effect of sperm concentration for sperm penetration, polyspermy, male pronucleus formation and number of penetrated sperm in the oocytes matured *in vitro*. Sperm concentrations were 0.2, 1, 5 and 10×10⁶/ml, respectively.

Experiment 3 was carried out to examine the effect of sperm concentration on developmental ability of pig embryos during *in vitro* culture. Sperm concentrations were 0.2, 1, 5 and 10×10⁶/ml, respectively.

Statistical analysis

Analysis of variance (ANOVA) was performed using the SAS software package (1996). Duncan's multiple range test was used to compare mean value of individual treatments, when the F-value was significant (p<0.05).

RESULTS

The percentage of sperm motility on the liquid boar semen diluted and preserved at 4°C with lactose hydrate, egg yolk and N-acetyl-D-glucosamine (LEN) diluent is presented in Table 1. There were significant differences on sperm motility according to preservation day and incubation

Table 1. Sperm motility (%) according to preservation day and incubation time

Day	Incubation time (h) ¹					
	0.5	2	3	4	5	6
1	96.9±1.9 ^{aw}	91.3±1.3 ^{ax}	86.9±1.2 ^{axy}	85.0±2.0 ^{ay}	83.1±1.2 ^{ayz}	78.8±1.3 ^{az}
2	85.8±3.3 ^{bw}	80.0±3.5 ^{bwx}	76.3±3.1 ^{bwxy}	72.5±3.2 ^{bxyz}	67.5±4.8 ^{byz}	61.3±4.3 ^{bz}
3	78.8±3.1 ^{bw}	73.0±2.7 ^{bwx}	70.0±4.1 ^{bwx}	62.5±4.3 ^{bxy}	55.0±3.5 ^{xy}	51.3±4.7 ^{xy}
4	63.8±2.4 ^{cw}	57.0±3.4 ^{cw}	43.8±3.8 ^{cx}	35.0±4.6 ^{cx}	22.5±3.2 ^{dy}	13.8±3.1 ^{dy}
5	46.3±3.8 ^{dw}	30.0±4.6 ^{dx}	18.8±4.3 ^{dy}	8.8±2.4 ^{dz}	6.3±1.3 ^{ez}	2.5±1.4 ^{ez}

¹ Mean±SE. Experiments were repeated six times.^{a, b, c, d, e} Values in the same column with different superscripts differ significantly ($p < 0.05$).^{w, x, y, z} Values in the same row with different superscripts differ significantly ($p < 0.05$).**Table 2.** Normal acrosome (%) according to preservation day and incubation time

Day	Incubation time (h) ¹					
	0	2	3	4	5	6
1	93.3±0.6 ^{aw}	90.0±1.8 ^{awx}	87.0±1.4 ^{ax}	80.5±1.7 ^{ay}	76.0±1.6 ^{ayz}	73.8±1.8 ^{az}
2	82.3±2.0 ^{bw}	73.5±2.5 ^{bx}	69.3±1.7 ^{bxy}	66.0±2.1 ^{by}	59.3±1.4 ^{bz}	56.8±1.4 ^{bz}
3	75.0±3.1 ^{bw}	40.8±2.8 ^{cx}	30.5±1.5 ^{cy}	21.8±2.8 ^{cz}	17.0±2.4 ^{cz}	15.3±1.9 ^{cz}
4	57.0±3.7 ^{cw}	37.5±1.0 ^{cx}	21.5±2.1 ^{dy}	15.0±2.1 ^{dz}	11.8±1.1 ^{dz}	8.5±1.7 ^{dz}
5	23.0±1.5 ^{dw}	21.0±2.2 ^{dw}	15.0±1.9 ^{ex}	5.8±0.9 ^{ey}	4.5±1.0 ^{ey}	2.5±1.2 ^{ey}

¹ Mean±SE. Experiments were repeated six times.^{a, b, c, d, e} Values in the same column with different superscripts differ significantly ($p < 0.05$).^{w, x, y, z} Values in the same row with different superscripts differ significantly ($p < 0.05$).**Table 3.** Effect of sperm concentration on fertilization parameters of pig oocytes matured *in vitro*

Sperm concentration ($\times 10^6$ /ml)	No. of oocytes inseminated	% of oocytes penetrated ¹	% of polyspermic oocytes ¹	% of oocytes with male pronucleus ¹	Mean no. of sperm in penetrated oocyte ¹
0.2	121	22.2±2.9 ^c	10.8±1.7 ^c	19.3±2.5 ^b	2.0±0.3 ^c
1	115	75.6±1.7 ^b	34.2±2.8 ^b	68.1±2.9 ^a	3.6±0.5 ^{bc}
5	140	80.1±0.4 ^a	82.2±3.3 ^a	70.6±2.0 ^a	5.8±1.0 ^b
10	153	86.7±2.8 ^a	85.7±2.7 ^a	71.0±4.0 ^a	9.3±0.8 ^a

¹ Mean±SE. Experiments were repeated four times.^{a, b, c} Values in the same column with different superscripts differ significantly ($p < 0.05$).

time, respectively. The sperm motility steadily declined from 96.9% at 0.5 h incubation to 78.8% at 6 h incubation at 1 day of preservation. However, the sperm motility rapidly declined after 4 day of preservation during incubation.

The percentage of normal acrosome on the liquid boar semen diluted and preserved at 4°C with LEN diluent is presented in Table 2. There were significant differences on normal acrosome according to preservation day and incubation time. The normal acrosome steadily declined from 93.3% at 0.5 h incubation to 73.8% at 6 h incubation at 1 day of preservation. However, the normal acrosome rapidly declined after 3 day of preservation during incubation.

As shown in Table 3, the rates of sperm penetration and polyspermy of the oocytes inseminated with liquid boar sperm preserved at 4°C were higher in 5 and 10×10^6 sperm/ml than in 0.2 and 1×10^6 sperm/ml. Male pronuclear formation was lower in 0.2×10^6 sperm/ml than in 1, 5 and 10×10^6 sperm/ml. Mean numbers of sperm in penetrated oocyte were highest in 10×10^6 sperm/ml compared with other sperm concentrations.

As shown in Table 4, the rates of blastocysts from the cleaved oocytes (2-4 cell stage) were highest in 1×10^6 sperm/ml compared with other sperm concentrations. Cell numbers per blastocyst were higher in 1×10^6 sperm/ml than in 0.2×10^6 sperm/ml.

DISCUSSION

We used the first diluent of the LEN diluents without glycerol (Yi et al., 2002) for liquid boar semen preservation at 4°C. It has been generally accepted that fresh ejaculated mammalian sperm acquire the ability for penetration of ooplasm after preincubation for a certain duration at high concentration of sperm. The preincubation period of fresh ejaculated boar sperm for successful fertilization was reported to be 4-8 h (Hamano and Toyoda, 1986; Suzuki et al., 1996). When frozen-thawed ejaculated boar sperm were used for IVF, however, sperm penetration was observed without any preincubation (Wang et al., 1991). The result of this study confirmed that the liquid boar sperm stored at 4°C could also fertilize without preincubation.

It was suggested by Watson (1995) that the frozen-

Table 4. Effect of sperm concentration on developmental ability of pig embryos¹

Sperm concentration ($\times 10^6$ /ml)	No. of embryos cultured ²	% of cleaved oocytes ³	% of blastocyst from cleaved oocytes ³	Cell no. per blastocyst
0.2	124	75.3 \pm 3.3	8.6 \pm 0.8 ^c	20.4 \pm 1.5 ^b
1	116	85.1 \pm 4.1	30.8 \pm 3.4 ^a	28.0 \pm 2.2 ^a
5	128	84.7 \pm 4.3	18.2 \pm 2.8 ^b	24.2 \pm 3.2 ^{ab}
10	114	86.1 \pm 2.8	17.5 \pm 2.1 ^b	23.0 \pm 2.0 ^{ab}

¹ Mean \pm SE. Experiments were repeated four times.

² Cultured oocytes were selected after *in vitro* fertilization (IVF).

³ Rates of cleavage and blastocyst were examined at 48 and 144 h after IVF, respectively.

^{a, b, c} Values in the same column with different superscripts differ significantly ($p < 0.05$).

thawed sperm has already initiated capacitation. In other words, membranes of frozen-thawed sperm are more similar to those of the unfrozen-capacitated sperm, and this similarity may correspond to acrosome reaction. It was also reported that capacitated sperm increased during cooling from room temperature to 5°C and that these capacitated sperm did not need preincubation after freezing and thawing (Watson, 1996). However, the mechanism resulting capacitation without preincubation time remains unknown (Martinez, 1996).

Coy et al. (1999) reported that the penetration rate and number of sperm cells per oocyte were dependent on the boar, maturation system and sperm concentration, but the rate of male pronuclear formation seemed to be influenced only by the boar and the maturation system but not by sperm concentration. Wang et al. (1991) reported that high penetration rates (85-89%) and increased incidence of polyspermy were obtained at 25-100 $\times 10^6$ sperm/ml by frozen-thawed ejaculated sperm. Nagai et al. (1984) reported that sperm concentration at insemination by epididymal or ejaculated boar sperm was 2 $\times 10^6$ cells/ml. Abeysdeera and Day (1997) reported that insemination with 1 $\times 10^5$ sperm/ml by frozen-thawed ejaculated sperm resulted in a 40% sperm penetration rate of oocytes with 16% polyspermy. Mean number of sperm per oocyte was 1.2 \pm 0.1. At 5 $\times 10^5$ and 1 $\times 10^6$ /ml, penetration rate (84-87%) and polyspermy (57-64%) increased, with no difference between the two concentrations. However, mean number of sperm per oocyte increased with increasing sperm concentration.

In this study, sperm concentrations at insemination with liquid boar sperm stored at 4°C affected the rates of sperm penetration, polyspermy, male pronuclear formation of oocytes and the mean number of sperm in the penetrated oocyte, and the rates of blastocysts from the cleaved oocytes. In conclusion, we found out that liquid boar sperm stored at 4°C in the lactose-egg yolk diluent with N-acetyl-D-glucosamine could be used for IVF of pig oocytes matured *in vitro*. Also, we recommend 1 $\times 10^6$ /ml concentration in mTBM fertilization medium for IVF of pig oocytes.

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