

***In vitro* Fertilization and Development of Pig Oocytes Inseminated with Boar Sperm by Different Sperm Washing Media after Thawing of the Frozen Straws**

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ABSTRACT : This study was carried out to investigate *in vitro* fertilization and development of *in vitro* matured pig oocytes inseminated with the Duroc boar sperm by different sperm washing media after thawing of the 5 ml frozen straws. Immature follicular oocytes (30-40) were transferred into each well of a Nunc 4-well multidish containing 500 µl mTCM199 maturation medium. The sperm rich portion of ejaculates was collected into a 250 ml insulated vacuum bottle and gradually cooled 22 to 24°C over a 2 h period. Semen was centrifuged at 800 g for 10 min and the seminal plasma discarded. Sperm were resuspended in a lactose-egg yolk and N-acetyl-D-glucosamine (LEN) diluent to contain 1×10^9 sperm/ml and cooled to 5°C over a 2 h period. Immediately before freezing, semen was rediluted with an equal volume of LEN+4% glycerol and packed into 5 ml straws. After thawing of the 5 ml straw, the 5 ml semen was diluted with 20 ml Beltsville thawing solution (BTS) at room temperature. Oocytes were inseminated with untreated (unwashed and nonpreincubated) or treated sperm (washed two times in BTS, mTLP-PVA and mTBM media, respectively and nonpreincubated) with 2×10^7 sperm concentration. Oocytes were cocultured for 6 h in 500 µl mTBM fertilization. At 6 h after IVF, oocytes were transferred into 500 µl NCSU-23 culture medium for further culture of 6 h. Sperm penetration, polyspermy and male pronuclear formation of oocytes at 12 h after IVF and developmental ability of oocytes at 48 h after IVF were evaluated. Sperm penetration rate, male pronuclear formation and rate of cleaved embryos were higher in the BTS, mTLP-PVA and mTBM treatments than the unwashed treatment ($p < 0.05$). The rate of blastocysts from the cleaved oocytes (2-4 cell stage) were higher in the mTLP-PVA treatment than in the unwashed, BTS and mTBM treatments. In conclusion, we recommend the washing of frozen-thawed sperm with mTLP-PVA medium before *in vitro* fertilization of oocytes in mTBM medium. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 2 : 164-167)

Key Words : IVF, Boar Sperm, Washing Media, 5 ml Frozen-straw, Oocytes

INTRODUCTION

Since Mattioli et al. (1989) succeeded in getting piglets from IVM/IVF oocytes in pigs, *in vitro* production of pig embryos have been developed gradually. However, efficient production of pig embryos through IVM/IVF techniques has been hampered by the high incidence of polyspermy and a low incidence of male pronuclear formation.

Deep frozen boar sperm had poorer motility, acrosomal morphology and viability than fresh sperm (Courten and Paquignon, 1985; Weitze et al., 1986; Clarke and Johnson, 1987; Almlid and Johnson, 1988; Almlid et al., 1989; Hofimo and Almlid, 1991), and the accompanying poor farrowing rates (40-50%) and low litter size have made frozen boar semen impractical for the commercial swine producer (Johnson, 1985; Almlid et al., 1987; Hofimo and Almlid, 1991; Crabo and Dial, 1992).

Several procedures using frozen-thawed boar semen have been reported for *in vitro* fertilization of *in vitro* or *in*

in vivo matured porcine oocytes. However, the results have not been consistent sufficiently because of day-to-day variations between ejaculates, even under identical laboratory conditions. Potential explanations may be due to boar effects, to the purity of the sperm rich fraction, and to different semen treatment protocols for IVF. Little is known about the physiology of sperm capacitation. Furthermore, adequate *in vitro* procedures to accomplish optimal sperm-oocyte interactions such as coordinated binding of spermatozoa to the zona pellucida are still not available.

This study was carried out to investigate *in vitro* fertilization and development of *in vitro* matured pig oocytes inseminated with the same boar sperm by different sperm washing media after thawing of the 5 ml frozen straws.

MATERIALS AND METHODS

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 and 0.1% BSA at 30-35°C. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3 to 6 mm in diameter) using a 20 gauge needle fixed to a 10 ml disposable syringe. COCs were

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Table 1. Effect of washing medium after thawing of 5 ml straw on fertilization parameters of pig oocytes in mTBM

Washing medium	No. of inseminated oocytes	% of sperm motility ¹		% of oocytes penetrated ¹	% of polyspermic oocytes ¹	% of oocytes with male pronucleus ¹
		0 h	6h			
Unwashed	100	50.0±1.9	29.3±4.4 ^a	35.0±1.4 ^c	4.2±0.8	30.0±1.4 ^b
BTS washing	100	42.9±3.4	16.4±1.4 ^b	45.8±0.8 ^b	3.3±1.7	39.2±0.8 ^a
mTLP-PVA washing	100	42.9±4.2	22.1±3.0 ^{ab}	51.7±0.8 ^a	2.5±1.4	43.3±1.7 ^a
mTBM washing	100	43.6±4.3	15.0±1.9 ^b	53.9±2.1 ^a	4.1±2.2	43.9±1.9 ^a

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

washed three times in mTLP-PVA and were washed two times 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. After about 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% CO₂ in air.

Frozen semen processing

Semen was collected from one Duroc boar twice a week. The sperm-rich fraction (30 to 60 ml) of ejaculate was collected into an insulated vacuum bottle. 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 the supernatant solution was 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 1.0×10^9 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 GmbH, 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 center of each straw and the straws were horizontally placed on an aluminum rack and set into a liquid nitrogen tank containing liquid nitrogen (LN). The straws were situated 5 cm above the LN, and kept at that level for 20 min before the straws were transferred into LN storage.

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. 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, 5% CO₂ in air. The dishes were kept in a CO₂ incubator until spermatozoa were added for insemination.

For IVF, one frozen 5 ml straw was thawed at 52°C in 40 sec and was diluted with 20 ml Beltsville thawing solution (BTS) at room temperature. Oocytes were inseminated with untreated (unwashed and nonpreincubated) or treated sperm (washed two times in BTS, mTLP-PVA and mTBM solution, respectively and nonpreincubated) with 2×10^7 /ml sperm concentration. At 6 h after IVF, oocytes were transferred into 500 µl NCSU-23 culture medium containing 0.4% BSA for further culture.

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 and cleaved oocytes under a phase-contrast microscope at $\times 400$ magnification. Blastocysts on day 6 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).

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS, 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

The effects of washing media after thawing of 5 ml straws on fertilization parameters were shown in Table 1. The present study showed that sperm motility reduced dramatically during culture in each treatment (about 42.9-50.0% at the start of culture to 15.0-29.3% at 6 h of culture). Oocytes inseminated with the mTLP-PVA and mTBM

Table 2. Effect of washing medium after thawing of 5 ml straw on development ability of pig oocytes after maturation and fertilization *in vitro*

Washing medium	No. of cultured oocytes ¹	% of cleaved oocytes ²	% of blastocysts from cleaved oocytes ²
Unwashed	74	54.1±2.7 ^b	9.5±1.4 ^b
BTS washing	76	65.9±1.8 ^a	7.9±0.2 ^b
mTLP-PVA washing	78	70.7±2.4 ^a	12.9±0.7 ^a
mTBM washing	83	71.1±2.8 ^a	7.2±0.1 ^b

¹ Cultured oocytes were selected after *in vitro* fertilization. ² Mean±SE. Experiments were repeated three times.

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

washing showed 51.7 and 53.9% sperm penetration, 2.5 and 4.1% polyspermy, and 43.3 and 43.9 male pronuclear formation, respectively. Sperm penetration and male pronuclear formation were higher in the BTS, mTLP-PVA and mTBM washing treatments than the unwashed treatment ($p < 0.05$).

As shown in Table 2, the percentage of cleaved oocytes was higher in the BTS, mTLP-PVA and mTBM washing treatments than the unwashed treatment, and the percentage of blastocysts from cleaved oocytes were higher in the mTLP-PVA washing treatment than the unwashed, BTS, and mTBM washing treatments.

DISCUSSION

In this study, the motility of frozen boar sperm after thawing was 42.9-50.0% in each treatment, but the motility reduced dramatically during culture. We found out that frozen-thawed boar sperm, even with a low motility, contributed to the penetration rates of oocytes. Watson (1995) reported that the cryopreserved sperm somehow led to acceleration of capacitation-like process. Martinez et al. (1996) indicated that, under the *in vitro* conditions studied, boar sperm underwent capacitation and a true acrosome reaction during co-incubation with oocytes even when not washed or preincubated. Polyspermic penetration has been the persistent problem in IVM-IVF systems (Funahashi and Day, 1997). Polyspermic penetration of oocytes *in vitro* was not due to delayed or incomplete cortical granule exocytosis (Wang et al., 1998) but more likely to a delayed zona reaction and/or simultaneous sperm penetration (Wang et al., 1999).

Funahashi and Nagai (2001) reported that adenosine increased the normal penetration of oocytes by frozen-thawed boar sperm, and that the number of partially acrosome-reacted sperm at insemination might be one of the primary causes of polyspermy in porcine IVF system. In this study, we found out extremely low incidence of polyspermic penetration. These results were consistent with the above reports of Martinez et al. (1996), Wang et al. (1998, 1999) and Funahashi and Nagai (2001).

In general, semen pellet and straw were thawed and washed three times in mBO medium (Wang et al., 1995), TALP medium (Rath and Niemann, 1997), Dulbecco's PBS

(Abeydeera and Day, 1997; Abeydeera et al., 1998), mTCM199 medium (Kikuchi et al., 1999; Yamauchi and Nagai, 1999) and BTS (Gandhi et al., 2001; Marchal et al., 2002). This study was the first to compare the *in vitro* fertilization and development of *in vitro* matured pig oocytes inseminated with the Duroc boar sperm by different sperm washing media after thawing of the 5 ml frozen straws. The sperm penetration, male pronuclear formation, and percentage of cleaved oocytes were higher in the BTS, mTLP-PVA and TBM washing treatments than the unwashed treatment. These results indicated that the sperm washing after thawing of 5 ml frozen-straw was an important factor for higher sperm penetration and male pronuclear formation. The removed egg yolk particles in the LEN diluent during washing may be one of factors to improve the sperm penetration and male pronuclear formation.

The percentage of cleaved oocytes was higher in the BTS, mTLP-PVA and mTBM washing treatments than the unwashed treatment. The percentage of blastocysts from cleaved oocytes was higher in the mTLP-PVA washing treatment than the unwashed, BTS and mTBM washing treatments. The above results demonstrated that the sperm washing media contributed to sperm penetration rate, monospermy, cleavage rate and blastocyst formation of oocytes.

In conclusion, we found out that the washing of frozen-thawed sperm with mTLP-PVA medium before *in vitro* fertilization of oocytes in mTBM medium was very important to improve the blastocyst formation of oocytes.

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REFERENCES

- Abeydeera, L. R. and B. N. Day. 1997. *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: Effect of BSA, caffeine and calcium. *Theriogenology* 48:537-544.
- Abeydeera, L. R., W. H. Wang, T. C. Cantley, A. Rieke and B. N. Day. 1998. Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after *in vitro*

- fertilization: relevance to intracellular glutathione. *Biol. Reprod.* 58:213-218.
- Almlid, T., S. E. Staven and L. A. Johnson. 1987. Fertility evaluation of the straw freezing technique for boar semen under practical artificial insemination conditions. *Zuchthygiene* 22:193-202.
- Almlid, T. and L. A. Johnson. 1988. Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws. *J. Anim. Sci.* 66:2899-2905.
- Almlid, T. L., R. N. Clarke, V. G. Pursel and L. A. Johnson. 1989. Effectiveness of *in vitro* methods for predicting *in vivo* fertilizing capacity of boar spermatozoa cryopreserved with 2 or 4% glycerol. *Zuchthygiene* 24:8-15.
- Clarke, R. N. and L. A. Johnson. 1987. Effect of liquid storage and cryopreservation of boar spermatozoa on acrosomal integrity and the penetration of zona-free hamster ova *in vitro*. *Gamete Res.* 16:193-204.
- Courtens, J. L. and M. Paquignon. 1985. Ultrastructural of fresh, frozen-thawed spermatozoa. In: *Deep Freezing of Boar Semen* (Ed. L. A. Johnson and K. Larsson). Swedish University of Agricultural Science, Uppsala, pp. 61-87.
- Crabo, B. G. and G. D. Dial. 1992. Artificial insemination in swine. *Vet. Clin. North Am. Food. Anim. Pract.* 8:533-544.
- Funahashi, H. and B. N. Day. 1997. Advances in *in vitro* production of porcine embryos. *J. Reprod. Fertil.* 52 (Suppl.):271-283.
- Funahashi, H. and T. Nagai. 2001. Regulation of *in vitro* penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. *Mol. Reprod. Dev.* 58:424-431.
- Gandhi, A. P., M. Lane, D. K. Gardner and R. L. Krisher. 2001. Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media. *Mol. Reprod. Dev.* 58:269-275.
- Hofino, P. O. and T. Almlid. 1991. Recent developments in freezing of boar semen with special emphasis on cryoprotectants. In *Boar Semen Preservation*, Vol. 2, Proceedings of the 2nd International Congress on Boar Semen Preservation. *Reprod. Domest. Anim.* 1:111-122.
- Johnson, L. A. 1985. Fertility results using frozen boar spermatozoa: 1970 to 1985 In: *Deep Freezing of Boar Semen* (Ed. L. A. Johnson and K. Larsson). Swedish University of Agricultural Science, Uppsala, pp. 199-222.
- Kikuchi, K., N. Kashiwazaki, T. Nagai, J. Noguchi, A. Shimada, R. Takahashi, M. Hirabayashi, M. Shino, M. Ueda and H. Kaneko. 1999. Reproduction in pigs using frozen-thawed spermatozoa from epididymal stored at 4°C. *J. Reprod. Dev.* 45:345-350.
- Marchal, R., J. Pelaez, M. Terqui and P. Mermillod. 2002. Effect of sperm survival and CTC staining pattern on *in vitro* fertilization of porcine oocytes. *Theriogenology* 57:1917-1927.
- Martinez, E. A., J. M. Vazquez, C. Matas, J. Gadea, M. I. Alonso and J. Roca. 1996. Oocyte penetration by fresh or stored diluted boar spermatozoa before and after *in vitro* capacitation treatments. *Biol. Reprod.* 55(1):134-140.
- Mattioli, M., M. L. Bacci, G. Galeati and E. Seren. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. *Theriogenology* 31:1201-1207.
- Rath, D. and H. Niemann. 1997. *In vitro* fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. *Theriogenology* 47:785-793.
- SAS Institute Inc. 1996. *SAS/STAT guide for personal computer* 6.12. SAS Inst. Inc., Cary, NC., USA.
- Wang, W. H., L. R. Abeydeera, L. R. Fraser and K. Niwa. 1995. Functional analysis using chlortetracycline fluorescence and *in vitro* fertilization of frozen-thawed ejaculated spermatozoa incubated in a protein-free chemically defined medium. *J. Reprod. Fertil.* 104:305-313.
- Wang, W. H., L. R. Abeydeera, R. S. Prather and B. N. Day. 1998. Morphologic comparison of ovulated and *in vitro* matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. *Mol. Reprod. Dev.* 49:308-316.
- Wang, W. H., Z. Machaty, L. R. Abeydeera, R. S. Prather and B. N. Day. 1999. Time course of cortical and zona reaction of pig oocytes upon intracellular calcium increase induced by thimerosal. *Zygote* 7:79-86.
- Watson, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* 7:871-891.
- Weitze, K. F., F. Fazano, I. R. Scheid, I. Wentz and D. Rath. 1986. Fertility of boar spermatozoa frozen in 5 ml macro tubes and 1 ml minitubes. In *Proceedings of the 9th Congress of the International Pig Veterinary Society*. Barcelona, 80.
- Yamauchi, N. and T. Nagai. 1999. Male pronuclear formation in denuded porcine oocytes after *in vitro* maturation in the presence of cysteamine. *Biol. Reprod.* 61:828-833.
- Yi, Y. J., Y. A. Kwon, H. J. Ko and C. S. Park. 2002. Effects of diluent component, freezing rate, thawing time and thawing temperature on acrosome morphology and motility of frozen-thawed boar sperm. *Asian-Aust. J. Anim. Sci.* 15(11):1553-1558.