

Comparison of Semen Characteristics, Frozen-Thawed Sperm Viability, Testosterone Concentration and Embryo Development between Yorkshire Boar A and B

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ABSTRACT : This study was carried out to compare the semen characteristics, frozen-thawed sperm viability and testosterone concentration and *in vitro* fertilization (IVF) and development of *in vitro* matured pig oocytes between two Yorkshire boars. Semen and blood samples were collected once per week from October to November 2002 from two adult Yorkshire boars at 18 months of age with 170 kg body weight. Sperm were deep frozen in 5 ml maxi-straws with lactose-egg yolk and N-acetyl-D-glucosamine (LEN) diluent and stored in liquid nitrogen. Blood samples were obtained at 10 a.m. by inserting a 21 gauge, hypodermic needle attached to 10 ml syringe into surface veins in the ear. The concentration of testosterone was determined by Competitive Enzyme Immunoassay. Ovaries were collected from prepubertal gilts at a local slaughter house. Cumulus oocyte complexes were aspirated from antral follicles (3 to 6 mm in diameter). The medium used for oocyte maturation was modified TCM 199. After about 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% CO₂ in air. For IVF, one frozen 5 ml straw was thawed at 52°C in 40 sec and was diluted with 20 ml Beltsville thawing solution at room temperature. Sperm were washed 2 times in mTLP-PVA and inseminated without preincubation after thawing. Oocytes were inseminated with 2×10⁷/ml sperm concentration. Oocytes were coincubated for 6 h in 500 µl mTBM fertilization medium. At 6 h after IVF, oocytes were transferred into 500 µl NCSU-23 culture medium for further culture of 48 and 144 h. There were no significant differences in the semen volume, motility, normal acrosome morphology and sperm concentration of raw semen between A and B of Yorkshire boar. However, motility and normal acrosome of boar A were higher than those of boar B at 0.5, 2, 3, 4, 5 and 6 h incubations of frozen-thawed sperm. Testosterone concentration (3.75 ng/ml) of boar A was higher than that (2.34 ng/ml) of boar B. The rate of blastocyst formation (15.1%) of boar A was higher than that (10.4%) of boar B. In conclusion, serum testosterone concentration of boar showed very important role for the frozen-thawed sperm viability and the blastocyst formation of pig oocytes matured *in vitro*. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 5 : 612-616)

Key Words : Sperm Viability, Testosterone, Embryo, Yorkshire, Boar

INTRODUCTION

Deep frozen boar sperm had poorer motility, acrosomal morphology and viability than fresh sperm (Clarke and Johnson, 1987; Hofmo 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; Almild et al., 1987; Crabo and Dial, 1992). However, procedures using frozen-thawed boar semen will be useful for *in vitro* fertilization (IVF) of *in vitro* or *in vivo* matured porcine oocytes. Several procedures using frozen-thawed boar semen have been reported for IVF of *in vitro* or *in vivo* matured porcine oocytes. However, the results have not been consistent sufficiently because of day to day variations between ejaculates, even under identical 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 evaluating method in estimating the fertilizing ability *in vitro* of the frozen-thawed sperm. Therefore, this study was carried out to compare the semen characteristics, frozen-thawed sperm viability and testosterone concentration, and *in vitro* fertilization and development of *in vitro* matured pig oocytes between Yorkshire boar A and B.

MATERIALS AND METHODS

Semen and blood collection

Semen and blood were collected one time per week from October to November 2002 from two adult Yorkshire boars at 18 months of age with 170 kg body weight. Boars were housed at Division of Animal Science and Resources, Chungnam National University. The filtered sperm-rich fraction was collected by the gloved-hand technique into a 250 ml insulated vacuum bottle. Blood samples were obtained at 10 a.m. by inserting a 21 gauge, hypodermic needle attached to 10 ml syringe into surface veins in the ear. Blood samples were allowed to clot at 4°C and serum was obtained by centrifuged for 15 min at 1,000 g. The serum was then stored at -20°C.

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Received May 2, 2003; Accepted January 26, 2004

Determination of semen volume and sperm concentration

Semen volumes were determined with a graduated cylinder. Sperm concentrations were estimated by a hemocytometer. The sperm-rich fractions of ejaculates with greater than 90% motile sperm and normal acrosome were used for frozen semen processing.

Frozen semen processing and sperm evaluation

The sperm-rich fraction 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 of LEN+4% glycerol diluent (the second diluent) was added to 1 volume of the cooled semen. Straws (Minitub GmbH, Germany) were immediately filled with 5 ml of semen and steel or glass balls were used to seal the end of the straws. The air bubble was adjusted to the center of each straw and the straws were horizontally placed on 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. Sperm motility and normal acrosome were evaluated by the method of Yi et al. (2002).

Analysis of serum testosterone

The concentration of testosterone was determined by Competitive Enzyme Immunoassay. Aliquots of 50 µl porcine serum were collected into a clean glass test tube. The aliquots were added 0.5 µl of diethyl ether and mixed thoroughly with a vortexer. To separate the layers, the ether was removed by freezing the aqueous layer in an ethanol/dry ice bath and decanted. The ether was placed into a clean test tube. The ether as evaporated by heating to 30°C with a gentle stream of dry nitrogen. The extract was dissolved in 50 µl of Enzyme Immunoassay (EIA) buffer. The EIA kit (Cayman Chemical, MI, USA) was rinsed three times with washing buffer and added 50 µl of testosterone standard and sample per well, respectively.

Testosterone acetylcholinesterase tracer and testosterone antiserum of 50 µl, respectively, were incubated on plate shaker for one hour at room temperature. The wells were emptied and rinsed five times with washing buffer. Ellman's reagent of 200 µl per well was added and developed the plate in one hour and read the plate at 405 nm by Microplate Reader (Molecular Device, USA).

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 and 0.1% BSA (w/v) 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 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.

***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 2 times 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, 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. Sperm were washed 2 times in mTLP-PVA and then resuspended with mTBM, and inseminated without preincubation after thawing. Oocytes were inseminated with 2×10^7 /ml sperm concentration. Oocytes were coincubated for 6 h in 500 µl mTBM. At 6 h after IVF, oocytes were transferred into 500 µl NCSU-23 culture medium containing 0.4% BSA for further culture of 48 and 144 h.

Examination of oocytes

At 48 h after insemination, oocytes were examined the 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

Table 1. Comparison of Yorkshire boar A and B on semen characteristics of sperm rich fraction

Yorkshire boar	Volume (ml)	Sperm concentration ($\times 10^8/\text{ml}$) ¹	Motility (%) ¹	Normal acrosome (%) ¹
A	54.9 \pm 5.9	12.0 \pm 1.0	92.1 \pm 1.0	94.7 \pm 1.1
B	45.2 \pm 3.3	10.5 \pm 0.6	91.3 \pm 1.4	94.9 \pm 0.5

¹ Mean \pm SE for 8 ejaculates from each of Yorkshire boar A and B.

Table 2. Comparison of Yorkshire boar A and B on post-thaw sperm motility (%) according to incubation time in 5 ml straw

Yorkshire boar	Incubation time (h) ¹					
	0.5	2	3	4	5	6
A	55.1 \pm 2.6 ^{ax}	49.6 \pm 1.6 ^{bx}	44.9 \pm 1.3 ^{bcx}	42.0 \pm 0.9 ^{cx}	32.9 \pm 1.5 ^{dx}	23.1 \pm 1.8 ^{ex}
B	46.5 \pm 1.5 ^{ay}	38.4 \pm 1.9 ^{by}	35.0 \pm 1.7 ^{bey}	32.5 \pm 2.2 ^{cy}	25.6 \pm 1.7 ^{dy}	14.1 \pm 1.7 ^{ey}

^{a, b, c, d, e} Means with different letters within the same row were significantly different ($p < 0.05$).

^{x, y} Means with different letters within the same column were significantly different ($p < 0.05$). ¹ Mean \pm SE for 16 replications.

Table 3. Comparison of Yorkshire boar A and B on post-thaw sperm NAR acrosome (%) according to incubation time in 5 ml straw

Yorkshire boar	Incubation time (h) ¹					
	0	2	3	4	5	6
A	51.8 \pm 1.2 ^{ax}	23.8 \pm 0.8 ^{bx}	12.4 \pm 0.8 ^{cx}	7.4 \pm 0.9 ^{dx}	3.6 \pm 0.5 ^{ex}	2.8 \pm 0.8 ^{ex}
B	43.2 \pm 2.1 ^{ay}	15.2 \pm 1.0 ^{by}	5.6 \pm 1.1 ^{cy}	4.0 \pm 0.8 ^{cdy}	1.2 \pm 0.4 ^{dy}	0.6 \pm 0.2 ^{ey}

^{a, b, c, d, e} Means with different letters within the same row were significantly different ($p < 0.05$).

^{x, y} Means with different letters within the same column were significantly different ($p < 0.05$). ¹ Mean \pm SE for 16 replications.

Table 4. Effect of boar and testosterone concentration on development ability of pig oocytes after maturation and fertilization *in vitro*

Yorkshire boar	Testosterone concentration (ng/ml) ¹	No. of cultured oocytes ²	% of cleaved oocytes ³	% of blastocyst from cleaved oocytes ³	No. of cells per blastocyst
A	3.75 \pm 0.31 ^a	231	78.1 \pm 1.8	15.1 \pm 0.6 ^a	26.0 \pm 2.1
B	2.34 \pm 0.22 ^b	223	75.2 \pm 2.7	10.4 \pm 1.2 ^b	24.4 \pm 1.4

^{a, b} Means with different letters within the same column were significantly different ($p < 0.05$).

¹ Mean \pm SE for 8 blood collections from each of Yorkshire boar A and B. ² Cultured oocytes were selected after *in vitro* fertilization.

³ Mean \pm SE. Experiments were repeated 8 times.

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 and Student's *t*-test were used to compare mean values of individual treatment, when the *F*-value was significant ($p < 0.05$).

RESULTS

Comparison of semen characteristics between Yorkshire boar A and B

As shown in Table 1, semen characteristics of sperm rich fraction were compared between Yorkshire boar A and B. There were no differences between Yorkshire boar A and B on the semen volume, sperm concentration, motility and normal acrosome.

Comparison of Yorkshire boar A and B on post-thaw sperm motility according to incubation time in 5 ml straw

As shown in Table 2, there were no differences between

Yorkshire boar A and B on post-thaw sperm motility according to incubation time. The sperm motility after 2, 3, 4 and 6 h incubations was decreased compared with that after 0.5 h incubation in the Yorkshire boar A and B.

Comparison of Yorkshire boar A and B on post-thaw NAR acrosome according to incubation time in 5 ml straw

This study showed that Yorkshire boar A was higher NAR acrosome than Yorkshire boar B from 0 to 6 h incubations as shown in Table 3. The NAR acrosome after 2, 3, 4, 5 and 6 h incubations was dramatically decreased compared with that after 0 h incubation in the Yorkshire boar A and B.

Effect of boar and testosterone concentration on development ability of pig oocytes

Effects of Yorkshire boars and testosterone concentrations on development ability of pig oocytes after maturation and fertilization *in vitro* are presented in Table 4. Serum testosterone concentration was higher in Yorkshire boar A than in Yorkshire boar B. Also, percentage of blastocyst from cleaved oocytes was higher in Yorkshire boar A than in Yorkshire boar B.

Table 5. Correlation between testosterone concentration and percentage of blastocyst from cleaved oocytes

Parameter	Testosterone concentration	Percentage of blastocyst
Testosterone concentration	1	0.92**
Percentage of blastocyst	0.92**	1

** p<0.01.

Correlation between testosterone concentration and percentage of blastocyst formation

As shown in Table 5, we tried to find out a correlation between testosterone concentration and percentage of blastocyst from cleaved oocytes. Testosterone concentration was correlated ($p<0.01$) with the percentage of blastocyst from cleaved oocytes.

DISCUSSION

Semen production of boars is influenced by many factors, such as breed, age, nutrition, environmental effects, health status and frequency of use, which result in great variations of semen characteristics (Hughes and Varley, 1980). In this study, there were no differences between Yorkshire boar A and B on semen volume and sperm concentration of sperm rich fraction. Also, sperm motility and normal acrosome of raw semen in the two Yorkshire boars did not differ significantly. Semen volume and sperm concentration of sperm rich fraction in the two Yorkshire boars were similar to the report of Chung et al. (1989).

Borg et al. (1993) reported that characteristics of sperm morphology did not differ among breeds of boar. Various studies have demonstrated the wide variability among boars in fertility rates obtained from their frozen semen (Larsson, 1976). Coster (1978) concluded from a study of 42 boars that freezability could not be predicted from fresh semen evaluation. Breed differences using frozen-thawed boar semen have been demonstrated (Paquignon and Courot, 1976; Johnson et al., 1981, 1982). Park and Yi (2002) reported that sperm motility and normal acrosome of raw semen in Duroc and Yorkshire boars did not differ significantly among spring, summer, autumn and winter. However, sperm motility and normal acrosome of frozen-thawed sperm were higher in spring season than in summer, autumn and winter. In this study, the post-thaw sperm motility and normal apical ridge (NAR) acrosome according to incubation time showed significantly different between Yorkshire boar A and B.

Park and Yi (2002) reported that serum testosterone concentrations in Duroc and Yorkshire boars were higher in spring than in summer, autumn and winter. Also, serum testosterone concentrations in spring and summer were higher in Yorkshire boars than in Duroc boars. In this study, serum testosterone concentration in autumn season was higher in Yorkshire boar A than in Yorkshire boar B.

Sellés et al. (2003) reported that the *in vitro* fertilization seemed to be a good tool to evaluate the quality of frozen-thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm freezing procedures, as it was the more precise evaluating method in estimating the potential fertilizing ability. In this study, percentage of blastocyst from the cleaved oocytes was higher in Yorkshire boar A than Yorkshire boar B. Also, testosterone concentration was correlated with the percentage of blastocyst from cleaved oocytes.

When serum testosterone concentration was higher in Yorkshire boar A than in Yorkshire boar B, the post-thaw sperm motility and NAR acrosome, and the percentage of blastocyst from cleaved oocytes were higher in Yorkshire boar A than in Yorkshire boar B. As a result of this study, we found out that serum testosterone concentration of boar was very important for the frozen-thawed sperm viability and the blastocyst formation of pig oocytes matured *in vitro*.

ACKNOWLEDGEMENTS

This work was supported by grant No. R11-2002-100-01000-0 from ERC program of Korea Science & Engineering Foundation.

REFERENCES

- 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.
- Borg, K. E., D. D. Lunstra and R. K. Christenson. 1993. Semen characteristics, testicular size, and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing and Minzhu boars. *Biol. Reprod.* 49:515-521.
- Chung, C. K., H. K. Kim, M. S. Ko, I. C. Kim, J. S. Choi, K. W. Lee, D. S. Son, H. Kim, S. H. Chee and C. S. Park. 1989. Studies on fertilizing capacity and survival of liquid boar semen in 5 ml maxi straws. *Kor. J. Anim. Sci.* 31(3):158-161.
- 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.
- Coster, G. E. 1978. Tiefgefrierkonservierung von Ebersamen in Kunststoffrohren. *In vitro* untersuchungen zur verfahrensverbesserung sowie besamungsergebnisse nach awendung unter schiedlicher inseminationsmedien und-techniken. Hannover, Tierarztl. Hochschule, Discussion.
- Crabo, B. G. and G. D. Dial. 1992. Artificial insemination in swine. *Vet. Clin. North. Am. Food. Anim. Pract.* 8:533-544.
- Hofmo, 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.

- Hughes, P. and K. Varley. 1980. Reproduction in the pig. Butterworths, London, pp. 187-195.
- Johnson, L. A., J. G. Albers, C. M. T. Willems and W. Subesma. 1981. Use of boar spermatozoa for artificial insemination. Part . Fertilizing capacity of fresh and frozen spermatozoa in sows on 36 farms. *J. Anim. Sci.* 52:1130-1136.
- Johnson, L. A., J. G. Albers and J. A. M. Arts. 1982. Use of boar spermatozoa for artificial insemination. Part . Fertilizing capacity of fresh and frozen spermatozoa in gilts inseminated either at a fixed time or according to Walsmeta readings. *J. Anim. Sci.* 54:126-131.
- 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.
- Larsson, K. 1976. Fertility of deep frozen spermatozoa at various intervals between insemination and induced ovulation: influence of boars and thawing diluents. *Acta. Vet. Scand.* 17:63-73.
- Paquignon, M. and M. Courot. 1976. Fertilizing capacity of frozen boar spermatozoa. In: *Proceedings of the Eighth International Congress on Animal Reproduction and Artificial Insemination* (Kracow), 1041-1044.
- Park, C. S. and Y. J. Yi. 2002. Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. *Anim. Reprod. Sci.* 73:53-61.
- SAS Institute Inc. 1996. *SAS/STAT guide for personal computer* 6.12. SAS Inst. Inc., Cary, NC., USA.
- Sellés, E., J. Gadea, R. Romar, C. Matás and S. Ruiz. 2003. Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. *Reprod. Dom. Anim.* 38:66-72.
- 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.