



Some Factors Affecting Freezing of Boar Semen in 5 ml Maxi-straws*

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ABSTRACT : A series of experiments were conducted to determine the suitable freezing and thawing temperatures for the freezing of boar semen in 5 ml maxi-straws. The ultrastructure, *in vitro* fertilization (IVF) and artificial insemination (AI) of frozen-thawed semen were also be evaluated. The 5 cm freezing height gave the best results not only in post-thaw motility rate (54.00%), but also in normal acrosome morphology rate (NAR) (80.23%). There was no significant difference in the post-thaw motility between different thawing temperatures and corresponding thawing times ($p>0.05$); the group of 52°C and 25 s gave the highest motility rate (45.00%). As a whole, not only from the motility but also the NAR, thawing at 42°C was better than the other two treatments. In the freezing packages, 5 ml maxi-straw gave a little lower mobility (40%), viability rate (49.58%), plasma membrane integrity rate (53.91%) and NAR (52.65%) than the 0.25 ml straw, but there was no significant difference between the two straw volumes ($p>0.05$). The IVF capacity of frozen-thawed semen in this experiment was similar to fresh semen. From ultrastructure observation, the main damage to boar spermatozoa after freezing was seen in the acrosome, such as swelling and formation of vesicles. After AI in recipient Shanghai White sows, frozen-thawed semen from 5 ml maxi-straws and pellets produced 72.2% and 80% conception rate and 7.8 and 8 litter sizes, respectively, and there was no significant difference between the 5 ml maxi-straw and the pellet ($p>0.05$). (**Key Words :** Boar, Semen, Freeze, 5 ml Maxi-straw)

INTRODUCTION

Frozen-thawed semen would benefit AI in the commercial pig breeding industry. In cattle, AI is the routine reproductive biotechnology which has been widely used for genetic improvement, and most of the semen is frozen. However, various biochemical and anatomical compartments in the spermatozoa cells may be altered during the freeze-thaw process, and current methods for freezing boar semen are unsatisfactory. There are many disadvantages that make frozen-thawed boar semen an uneconomical option for commercial AI, such as the poor motility, acrosomal morphology and viability of frozen-

thawed semen (Waston, 1995; Zhou et al., 2004), low fertility levels (Johnson et al., 1981; Almlid et al., 1987; Cheon et al., 2002; Yi et al., 2004), requirements for a high number of spermatozoa per insemination dose ($5-6 \times 10^9$) compared with liquid semen ($2-3 \times 10^9$), between-boar variation in freezing success and the critical timing for AI (Amann, 2005). Consequently, the most important task before researchers is to establish a suitable boar sperm-freezing protocol to prevent the formation of lethal intracellular ice crystals and membrane damage during and after freezing.

When boar spermatozoa are subjected to freezing temperatures, extensive damage occurs between 0 and -20°C, a range of temperatures that has to be crossed twice during the freeze-thawing process (Bwanga, 1991). Because of this, semen samples can benefit from a fast temperature change and a fast heat transfer throughout the sample. During the last thirty years, several studies have optimized the freezing diluents of boar semen and the freezing protocols as well as the shape and the volume of the container (Salamon et al., 1973; Westendorf et al., 1975; Bwanga et al., 1990). Due to the large volume needed for successful porcine AI, 5 ml maxi-straws have become the

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most widely used packages (Almlid et al., 1996), even though the slow heat transfer can induce functional and biochemical damage to the sperm (Bwanga et al., 1990) and injuries can be reduced by lowering the straw volume (Weitze et al., 1987). Several authors have investigated the effect of different thawing procedures on boar semen packaged in 5 ml maxi-straws (Westendorf et al., 1975; Pursel et al., 1987). Maxi-straws have been thawed at temperatures ranging from 35°C to 90°C and for times ranging from 120 s to 6 s (Westendorf et al., 1975; Pursel et al., 1987; Corcuera, 1996; Eriksson et al., 2000).

Successful IVF can be induced effectively with both fresh sperm (Nagai et al., 1984) and frozen-thawed sperm (Nagai et al., 1988; Sellés et al., 2003). Pre-incubation in a suitable medium before IVF was important for capacitation of fresh sperm. However, frozen-thawed boar sperm were capable of IVF not only after pre-incubation (Nagai et al., 1988), but also without pre-incubation (Wang et al., 1991). The destruction of acrosome membrane may induce this phenomenon. Sellés et al. (2003) also reported that the IVF system was a good tool to evaluate the quality of frozen-thawed boar semen prior to its commercial use and a good way to assay new sperm freezing procedures.

All these above studies focused on the evaluation of motility, acrosome morphology and membrane integrity to assess semen quality, but less systematic study of 5 ml maxi-straws has been done. This study was carried out to establish a suitable 5 ml maxi-straw freezing procedure, including freezing, thawing, IVF and AI evaluation, and ultrastructure observation.

MATERIALS AND METHODS

Animals

Three 3-year-old mature boars (Shanghai White pig) with normal semen quality and proven fertility were selected as semen donors and were kept in individual pens. Shanghai White sows were used for AI, individually housed in straw-bedded pens with optimal ventilation, and were fed twice daily with water being provided *ad libitum*.

Semen collection and processing

Sperm-rich ejaculate fractions were obtained using the gloved-hand method. Then, the semen was extended (1:1(vol/vol)) in BTS. After collection, semen was maintained at room temperature for approximately 2 h after collection while macroscopic and microscopic characteristics were evaluated. Only ejaculate sperm samples with more than 90% motile spermatozoa were used. After centrifugation at 800×g for 10 min, the pellets were diluted in the first extender (11% lactose, 20% egg yolk, 0.1% N-acetyl-D-glucosamine) to a concentration of $1,500 \times 10^6$ cells/ml. After further cooling to 5°C in 2 h,

diluted spermatozoa were re-suspended with the second extender (11% lactose, 20% egg yolk, 4% glycerol and 0.5% Orvus Ex Paste) to a final concentration of $1,000 \times 10^6$ cells/ml. Diluted and cooled spermatozoa were placed in a refrigerator (5°C) for 2 h.

Semen freezing

5 ml and 0.25 ml straws : The 5 ml and 0.25 ml straws were filled and sealed manually, using metallic sealing balls and polyvinyl powder, respectively. 5 ml maxi-straws were wiped dry and the 3 cm air bubble was brought to the centre of the straw. 5 ml and 0.25 ml straws were placed in contact with nitrogen vapor for 20 min and 10 min, respectively, and then plunged into the liquid nitrogen tank and stored until usage.

Pellet freezing : After equilibration in a refrigerator at 5°C for 2 h, sperm suspensions were frozen in 0.2-ml pellets on a copper network (1 cm above the liquid nitrogen level). The sperm pellets were held for 30 s on the copper network and were transferred into the liquid nitrogen tank for storage.

Semen thawing

5 ml straws : Thawing was carried out in a water bath at a particular temperature for certain times following the experimental design. Thawed spermatozoa were extended at 37°C with BTS (1:1) and incubated for 30 min for immediate use. Frozen-thawed semen was partly assessed for motility, plasma membrane integrity, acrosome morphology, IVF, AI and ultrastructure observation.

0.25 ml straws : 0.25 ml straws were thawed in a water bath at 52°C for 12 s. Motility, viability, plasma membrane integrity and acrosome morphology were immediately assessed.

Pellet thawing : Each pellet of frozen spermatozoa that was removed from the liquid N₂ was placed into an aluminum box in a water bath at 52°C for 5 s, then plunged into a plastic tube containing 1 ml of BTS (1:1) pre-warmed to 37°C.

Spermatozoa cell counts

Spermatozoa cell concentration was assessed on fresh semen in 3% NaCl solution (1:20) using a Bürker chamber.

Assessment of sperm motility

Sperm motility was evaluated using a phase contrast microscope by placing a drop of the sample, without further dilution, on a microscope slide at 38°C and covering with a cover slip. Samples were evaluated at 400×magnification by estimating progressively motile spermatozoa (Berger et al., 1985). Motility was expressed as the percentage of motile spermatozoa per 100 sperm cells.

Assessment of the normal acrosome rate

Briefly, semen was washed in 0.9% NaCl solution by a brief centrifugation (10,000 rpm, 15 s) and fixed in 3.7% paraformaldehyde/PBS for 30 min. Samples were suspended in PBS followed by brief centrifugation, and spread on slides for air drying. The sperm smear was stained in 0.22% Coomassie blue G-250 for 5 min and washed in H₂O. Air-dried slides were mounted and then checked for the percentage of acrosome-intact spermatozoa in at least 200 spermatozoa under a bright field.

Plasma membrane integrity

An easily identifiable swelling and coiling of the tails occurred when boar spermatozoa were incubated at 37°C for 60 min in a mixture of 1.35% fructose and 0.73% Na-citrate. Plasma membrane integrity was expressed as the percentage of spermatozoa with swelling and coiling tails per 100 sperm cells.

Preparation of ultrastructure samples

Briefly, sperm samples were mixed with 2.5% glutaraldehyde solution for 2 h, then were washed in PBS for three times and mixed with 1% osmium tetroxide. After fixing the sample with osmium tetroxide, samples were washed three times in PBS and dehydrated with a series concentration of ethanol. Samples were then embedded in 618 Solution for 1 h. Ultrasections were stained with uranyl acetate, and were examined under a transmission electron microscope (Medical College electron microscope laboratory, Fudan University).

In vitro fertilization

For IVF, swine ovaries were obtained from a local slaughterhouse and transported to the laboratory where oocytes, with cumulus cells, were aspirated from 3 to 8 mm diameter follicles. About 80 oocytes were cultured in a 400 µl drop of maturation medium (TCM 199 medium supplemented with 69 µg/ml L-cysteine, 10% porcine follicular fluid, 10 IU/ml PMSG, 10 IU/ml HCG and 1 µg/ml β-estradiol) in a humidified atmosphere with 5% CO₂ at 38.5°C for 44 h. Matured oocytes were treated with 0.1% (w/v) hyaluronidase for 5 min, pipetted to strip away cumulus cells, and washed two to three times with TCM199 and modified Tris-buffered medium (mTBM). Fifteen to twenty oocytes were placed in a 50 µl drop of pre-equilibrated mTBM covered with mineral oil at 38.5°C in 5% CO₂ in air. Fresh or frozen-warmed boar spermatozoa were washed twice with Dulbecco's phosphate buffered saline (DPBS) and one to two times with mTBM followed by centrifugation at 1,500 g for 5 min each. The resulting fresh spermatozoa were re-suspended at 1.0×10⁶ cells/ml and frozen-thawed spermatozoa were re-suspended at 1.0×10⁷ cells/ml in mTBM containing 100 mg/ml heparin.

Subsequently, 50 µl spermatozoa were added to 50 µl mTBM containing the oocytes. After co-culture for 6-8 h, the presumptive zygotes were washed two to three times in NCSU-23 medium supplemented with 4 mg/ml BSA (Sigma), cultured up to 7 days in equilibrated NCSU-23 medium at 38.5°C in a humidified atmosphere of 5% CO₂ in air (Wu et al., 2006), and examined daily under an inverted microscope.

Artificial insemination (AI)

In vivo fertility study was conducted at the pig farm of Shanghai Academy of Agricultural Science. Estrus was checked daily in the presence of a mature teaser boar and occurrence of estrus was defined by the standing reflex in front of the boar (back pressure test) and reddening and swelling of the vulva. The sows were inseminated with diluted semen (about 30×10⁸ spermatozoa), using disposable AI-catheters. Insemination took place 12 h after diagnosis of estrus and was repeated 12 h later.

Experimental designs

Experiment 1 was conducted to study the effect of distance of straws from the liquid nitrogen surface on frozen-thawed sperm quality in 5ml maxi-straws. Experiment 2 was carried out to investigate the effect of thawing temperatures on sperm quality in 5 ml maxi-straws. Experiment 3 was undertaken to explore whether different freezing packages could affect frozen-thawed sperm quality. Experiment 4 was performed to evaluate the influence of deepfreeze on *in vitro* developmental ability after IVF. Experiment 5 was conducted to investigate the effect of deepfreeze on changes of spermatozoa ultrastructure. Experiment 6 was designed to study the effect of AI.

Statistical analysis

Experiments were replicated three times or more. Analyses of variance (ANOVA) were carried out using the SAS package (1988) in a completely randomized design. Duncan's multiple range test was used to compare mean values of individual treatments, when the F-value was significant or highly significant ($p < 0.05$ or $p < 0.01$).

RESULTS

Effect of straw distance from liquid nitrogen surface on post-thaw motility, plasma membrane integrity rate and NAR of boar sperm after freezing of 5 ml maxi-straws

In this experiment, post-thaw motility and acrosome morphology of boar sperm at 1, 3, 5, 7, 9 and 11 cm freezing height from liquid nitrogen were compared. The 5 cm freezing height group produced the best result not only in post-thaw motility rate (54.00%), but also in NAR (80.23%), and was significantly better than the 11 cm

Table 1. Effect of aluminum rack distance from liquid nitrogen surface on post-thaw motility, plasma membrane integrity rate and acrosome morphology of boar sperm after freezing of 5 ml maxi-straws

Freezing height (cm)	Post-thaw motility rate (%)	Plasma membrane integrity rate (%)	NAR (%)
1	41.67±2.89 ^{ABab}	46.45±8.60	60.08±1.05 ^{Bed}
3	48.33±7.64 ^{ABa}	46.94±5.67	68.69±4.99 ^{ABbc}
5	54.00±1.41 ^{Aa}	50.18±3.29	80.23±1.05 ^{Aa}
7	48.33±10.41 ^{ABa}	41.86±4.00	65.95±7.68 ^{Be}
9	45.00±5.00 ^{ABab}	45.95±3.96	61.12±3.46 ^{Bed}
11	35.00±5.00 ^{Bb}	50.98±3.83	55.54±1.83 ^{Bd}

Means with different small letters within the same columns were significantly different ($p < 0.05$); means with different capital letters within the same columns were highly significantly different ($p < 0.01$).

freezing height group ($p < 0.01$). There were no significant differences in the plasma membrane integrity rate after warming ($p > 0.05$).

In this experiment, temperatures of 5 ml maxi-straws were also measured in the process of freezing. The cooling rates were increased slightly following the reduction of freezing height (Data are not listed).

Effect of different thawing temperatures and times on frozen boar sperm

Using lactose-egg yolk (LEY) with glycerin as diluent for the freezing of boar sperm, three thaw temperatures and times (42°C for 30 s, 40 s, 50 s; 52°C for 18 s, 25 s, 35 s, 45 s and 60°C for 20 s, 30 s, 40 s) were compared in this experiment.

As shown in Table 2, there was no significant difference in post-thaw motility between different thaw temperature and its corresponding thaw time ($p > 0.05$). The highest motility rate (45.00%) was obtained in 52°C for 25s. For the rate of normal acrosome morphology, the group of 42°C for 40 s had the highest NAR (59.5%) and the group of

52°C for 18 s (28.78%) was the lowest. As a whole, not only for motility but also for normal acrosome morphology, 42°C thaw temperature was better than 52°C or 60°C.

Effect of different freezing packages on boar sperm freezing

Using LEY as the dilution solution, the freezing effect was compared in pellet, 0.25 ml straw and 5 ml maxi-straw in this experiment.

From Table 3, this experiment indicated that plasma membrane integrity and NAR of 0.25 ml straws after thawing were significantly higher than the pellet method ($p < 0.05$). There was no significant difference in mobility rate among the three groups ($p > 0.05$). The plasma membrane integrity rate and NAR after thawing of 5 ml maxi-straw were a little lower than 0.25 ml straw but were higher than the pellet method.

The probe of low temperature thermometer was put on the same level as the straws in the process of freezing, and temperatures were recorded every minute. After equilibrium in liquid nitrogen before freezing, initial temperatures of 5

Table 2. Effect of different thawing temperatures on post-thaw motility, plasma membrane integrity and NAR of boar sperm after freezing in 5 ml maxi-straws

Thaw temperature (°C)/thaw time (s)	Post-thaw motility rate (%)	Plasma membrane integrity (%)	NAR (%)
42/30	41.67±2.89 ^a	42.69±8.60 ^{ABb}	53.33±7.02 ^{ABa}
42/40	41.67±2.89 ^a	51.36±7.07 ^{Aa}	59.50±11.95 ^{Aa}
42/50	35.67±4.04 ^a	51.45±2.69 ^{Aa}	47.36±11.97 ^{ABab}
52/18	40.00±8.66 ^a	51.27±4.90 ^{Aa}	28.78±2.39 ^{Bb}
52/25	45.00±13.23 ^a	54.35±8.14 ^{Aa}	45.83±3.33 ^{ABab}
52/35	30.00±5.00 ^b	51.49±2.79 ^{Aa}	35.67±6.64 ^{Bb}
52/45	35.00±5.00 ^a	51.31±2.69 ^{Aa}	52.00±2.50 ^{ABa}
60/20	41.67±7.64 ^a	34.01±7.73 ^{Bb}	29.52±7.05 ^{Bb}
60/30	34.25±5.06 ^a	43.76±6.24 ^{ABab}	30.88±14.06 ^{Bb}
60/40	37.50±10.61 ^a	53.86±5.88 ^{Aa}	32.36±1.61 ^{Bb}

Table 3. Results of boar semen freezing in pellet, 0.25 ml straw and 5 ml maxi-straw

Freezing package	Post-thaw motility rate (%)	Plasma membrane integrity (%)	NAR (%)
Pellet	39.00±4.00	47.42±3.67 ^b	48.18±7.37 ^b
0.25 ml straw	46.00±4.00	57.91±3.74 ^a	59.86±2.29 ^a
5 ml maxi-straw	40.00±6.00	53.91±4.11 ^{ab}	52.65±5.13 ^{ab}

Means with different small letters within the same columns were significantly different ($p < 0.05$).

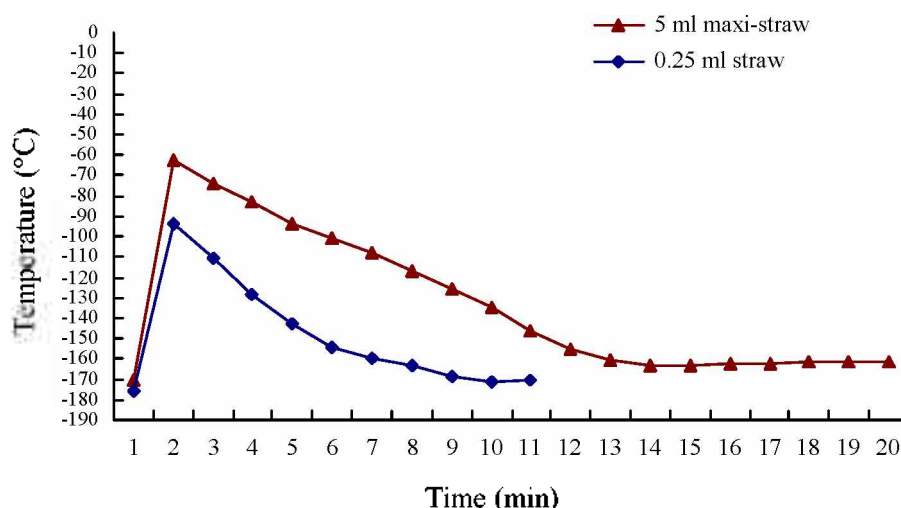


Figure 1. The freezing curve of boar semen frozen in 0.25 ml and 5 ml straws.

Table 4. Comparison between frozen and fresh semen of the developmental ability after IVF

Source of semen	No. of Oocytes	2-cell rate (%)	8-cell rate (%)	Morula rate (%)	Blastocyst rate (%)
Fresh semen	112	72.3 (81/112)	55.4 (62/112)	37.5 (42/112)	2.68 (3/112)
Frozen-thawed semen	110	74.5 (82/110)	59.1 (65/110)	36.4 (40/110)	1.82 (2/110)

ml maxi-straws and 0.25 ml straws were -170.2°C and -175.6°C , respectively. When samples were suspended in liquid nitrogen vapor, the temperature climbed quickly and reached -62.4°C and -93.3°C , respectively. Figure 1 indicates that cooling rate of the 0.25 ml straw was faster than that of the 5 ml maxi-straw. About seven and twelve minutes later, 0.25 ml and 5 ml straws reached relatively stable temperatures.

IVF of frozen-thawed boar semen packaged in 5 ml maxi-straw

IVF capacity of frozen-thawed semen from 5 ml maxi-straws was compared to fresh semen. The sperm

concentration of fresh sperm in IVF was 1×10^6 and frozen-thawed was 1×10^7 ; the co-incubation time of sperm-oocytes was 6 h. The IVF results of frozen-thawed semen for 2-cell, 8-cell, morula and blastocyst rates were 74.5%, 59.1%, 36.4% and 1.82%, respectively. There were no significant differences between the two groups ($p > 0.05$).

The ultrastructure change of frozen-thawed sperm

In fresh sperm, the plasma membrane, inside and outside membrane of the acrosome and nuclear membrane, were all integrated (Figure 2); the plasma membrane of the sperm head was closely positioned to the outside membrane of the acrosome (Figure 2). Very few fresh sperms had an

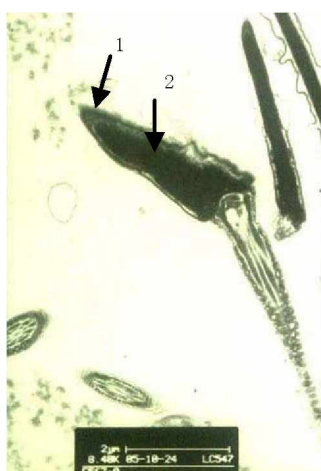


Figure 2. Head and body of fresh sperm (10,000 \times). 1. acrosome membrane, 2. nuclear.



Figure 3. Head of fresh sperm (10,000 \times). 1. 2 plasma membrane.

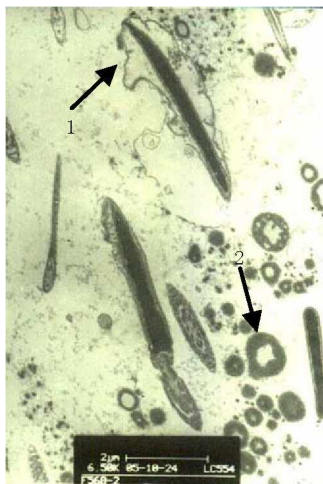


Figure 4. Head of the frozen-thawed sperm (10,000 \times). 1. acrosome membrane; 2. egg-yolk.

expanded plasma membrane and some vesicles around the outer acrosome membrane (Figure 3).

After freezing, the ultrastructure of frozen-thawed boar sperm had many differences to fresh sperm. The inside and outside membrane of the acrosome showed swelling. The number of vesicles in the outside membrane of the acrosome increased and were centered in the foreside of the acrosome (Figure 4). Some plasma membranes of frozen-thawed sperm had very big vesicles because of excessive swelling (Figures 4 and 5). Some acrosomes had granules under the outside plasma membrane (Figure 5). As there was swelling in the inside and outside membrane, the integrity of the membrane was very poor and materials in the acrosome were leaked (Figure 5).

Artificial insemination

Shanghai White boar thawed semen from 5 ml maxi-straws and pellets were applied to the artificial insemination. Using thawed semen of 5 ml maxi-straws and pellets, 72.2% and 80.0% pregnancy rate and 7.8 and 8.0 litter sizes were obtained, respectively, in recipient Shanghai White sows. There were no significant differences between 5 ml maxi-straws and pellets ($p>0.05$).

DISCUSSION

As previously known, the majority of cellular damage is thought to occur when cooling between 0°C to -20°C which the freezing and thawing process must pass through twice, while only 15-25% of sperm damage occurs at temperatures below -20°C (Pursel et al., 1985). Large and stable ice



Figure 5. Head of the frozen-thawed sperm (10,000 \times). 1. 2 plasma membrane.

crystals form in the freezing process and recrystallization during the thaw can damage the sperm plasma membrane and mitochondria (Courstens et al., 1985; Fiser et al., 1990; Thomas et al., 1998). On one hand, a decrease in the freezing and thawing velocity reduces ice crystal formation, but increases toxicity action because of the long contact time between cryoprotectant and semen; on the other hand, a fast velocity induces the abrupt change of osmotic stress and causes unbalanced rates of water outflow and influx, which can lead to shrinkage or swelling and lysis of spermatozoa (Mazur et al., 1984). Former studies showed that the optimal freezing speed in a 0.5 ml straw was 30°C/min in 3% glycerol (Fiser, 1990), in a 0.25 ml straw was 50°C/min in 1.5% glycerol (Woelders et al., 1993), and in a 5 ml maxi-straw was 16°C/min in 3.3% glycerol (Pursel et al., 1987). Due to their low tolerance to glycerol, the freezing of boar spermatozoa has to be relative rapid. Eriksson et al. (2000) indicated that the best post-thaw motility, when using 5 ml packages, was obtained with a freezing rate of 50°C/min (chamber temperature) with 3% glycerol, although the absolute differences were rather small when compared with samples frozen at 20°C/min or 80°C/min.

The aluminum rack distance from the liquid nitrogen surface in the cryopreservation can affect the cooling rate of the semen sample significantly. It was reported that the freezing and thawing rates in 1.8 ml and 0.5 ml straws were similar, from 4°C to -20°C at 30°C/min; from -20°C to -100°C at 92.7°C/min and 82.4°C/min. However, the 5 ml straw was different from these outcome. When the distance was 2 cm from the 5 ml-straw to the liquid nitrogen, the

Table 5. Results of the artificial insemination

Freezing method	Recipient sows	Number of recipient sows	Pregnancy rate (%)	Litter size
5 ml maxi-straw	Shanghai White sow	36	26/36 (72.2)	7.8
Pellet	Shanghai White sow	20	16/20 (80)	8.0

freezing rate (4°C to -20°C at 11°C/min; -20°C to -100°C at 36.6°C/min) was slower than that for 1 cm (4°C to -20°C at 15.4°C/min; from -20°C to -100°C at 51.2°C/min) (Cabrita et al., 2001). In this study, we found that the 5 cm height produced better results in the post-thaw motility and normal acrosome morphology rate (54.00% and 80.23%, respectively) than the 1 cm height (41.67%, 60.08%) and the 11 cm height (35.0%, 55.54%), which were consistent with the above studies. The improvement in the motility and NAR at 5 cm height could be attributed to the feasible freezing height, which reduced ice crystal formation and cell damage.

The rate of thawing through the critical temperature range is an important factor affecting survival of spermatozoa. In boar semen frozen at the optimum rate, both sperm motility and NAR are improved with an increasing thawing rate (Salamon et al., 1973; Pursel et al., 1975; Fiser et al., 1993). There is a threshold value in thawing rate where the risk of recrystallization of minute ice crystals during thawing is minimized. Eriksson et al. (2000) showed that the best thawing regime for Flatpacks, in terms of post-thaw motility, was 50°C for 13 s, and sperm survival could not be further improved by using an even higher thawing rate, suggesting that there is a point at which thawing rate and sperm survival balance each other. This is in agreement with what has been found by others when freezing boar (Fiser et al., 1993), bull (Woelders et al., 1998) and ram semen (Söderquist et al., 1997).

Cordova-Izquierdo et al. (2006) showed that thawing straws at 42°C for 40 s significantly reduced motility compared to straws thawed at 50°C for 40 s. This study also obtained the highest motility rate in thawing conditions of 52°C for 25 s, but for NAR, 42°C groups were better than the other two temperature groups. Using relatively low thawing temperature of 42°C, increasing thawing time did not improve motility rate. When we thawed at 52°C or 60°C, the motility of sperm just after thawing was relatively high, but survival time was relatively short and the motility of sperm was reduced to below 30% within 1 h. We speculate that higher temperature may induce greater damage to the sperm acrosome and plasma membrane morphology.

Pellets, 0.25 ml and 5 ml straws all have their own advantages and disadvantages. The volume of the 5 ml maxi-straw was larger than that of the pellet and the 0.25 ml straw, and, even if the heat transfer rate was low and there was a big temperature difference between the centre and periphery, freezing and thawing in 5 ml maxi-straws may induce more injury (Courtens et al., 1985; Fiser et al., 1990). The dose required for AI in the pig is very large, and the 0.25 ml straw and the pellet have great limitations and are inconvenient in practical application. Many researchers have favored 5ml maxi-straws in the past 10 years (Almlid

et al., 1996). Previous studies showed that there were no significant differences between the 0.5 and 5 ml straws for polyspermy in IVF (Cordova-Izquierdo et al., 2006). The results of this study suggested that plasma membrane integrity rate and NAR of sperm in the 0.25 ml straw were higher than the 5 ml maxi-straw ($p < 0.05$), and the motility and viability rate in the pellet, 0.25 ml and 5 ml straws were not significantly different ($p > 0.05$). These results were very similar to the above previous findings.

In order to simplify the source of semen for IVF and avoid the effects by different boars and different semen collection times, many laboratories now use frozen-thawed spermatozoa to perform the IVF (Abeydeera et al., 1997; Gil et al., 2003). Equally, IVF has been shown to be a good tool for evaluating the fertilizing capacity of frozen boar semen (Hammit et al., 1989; Selles et al., 2003) and indicated that it would be very useful for evaluating freezing procedures (Watson, 1995; Cordova et al., 2001; Selles et al., 2003). In this study, there was no significant difference between frozen-thawed semen and fresh semen in developmental capacity *in vitro* after IVF ($p > 0.05$). Also in AI, a 72.2% pregnancy rate was obtained in recipient Shanghai White sows by frozen-thawed semen from 5 ml maxi-straws. Through *in vitro* and *in vivo* fertilization experiments, good spermatozoa quality after warming was identified and relative satisfactory freezing and thawing processes were established.

Semen cryopreservation depends on cryobiology. Cryopreservation is a double-edged sword, which may protect or injury organisms. Mammalian sperm is a specialized cell, which contains concentrated chromatin. DNA damage in frozen-thawed semen was very common, and had been proved by the analysis of flow cytometry and trials of single-cell gel electrophoresis (Royere et al., 1991; Jiang et al., 2007). Previous studies showed that a sperm capacitation phenomenon could be seen in frozen-thawed sperm, which mainly focused on the swelling sperm membrane and acrosome membrane (Watson, 1995). Leakage of sperm acrosome contents, inflated sperm plasma membrane and a bubbly sperm acrosome membrane were also seen in this study. The vesicles in the sperm head were more serious than that in the tails. It is probably because the sperm tail is enclosed by the fibrous sheath, that damage does not easily occur. We also found that several granules of high electron concentration existed in the acrosomes of frozen-thawed spermatozoa. Physical or chemical changes to the character of the materials in the acrosome may be the reason for this phenomenon and further relevant research needs to be conducted.

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