The Establishment of Boar Semen Cryopreservation in Thailand:
Post-thaw Semen Quality, Sperm Concentration
and Variation among Ejaculates

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Abstract

The present study aimed at establishing boar semen cryopreservation in Thailand. Fresh and post-thaw semen quality, sperm concentration and variation among ejaculates were investigated. Forty-five ejaculation of semen from 15 boars were collected. The semen was diluted with an isothermal Beltsville thawing solution (BTS) extender, held at 15°C for 2 h and centrifuged. The semen precipitant was re-suspended with lactose-egg yolk (LEY) extender. The diluted semen was cooled down to 5°C within 90 min. Two parts of semen were mixed with one part of LEY extender containing 9% glycerol and 1.5% Equex-STM Paste. The extended semen samples were loaded into 0.5 mL straws. The straws were placed on liquid nitrogen (LN2) vapor for 20 min, then plunged into LN2 and frozen. Thawing was conducted by immersing the straws in water at 50°C for 12 sec. Sperm concentration, sperm motility, sperm viability, percentage of normal apical ridge (NAR) acrosomes, sperm plasma membrane function (sHOST) and sperm plasma membrane integrity (SYBR) were evaluated. On average, the sperm concentration post-thaw was 811x10^6 spermatozoa mL^-1. The motility, the viability, the NAR, the sHOST and the SYBR of boar spermatozoa post-thaw were 28%, 36%, 26%, 19% and 31%, respectively. The motility of frozen-thawed spermatozoa significantly correlated with the sperm viability (P<0.001), the NAR (P<0.02), the sHOST (P<0.001) and the membrane integrity (SYBR) (P<0.001). The higher concentration of frozen-thawed semen resulted in the lower membrane integrity (r=-0.3, P=0.04). Post-thaw sperm concentration was significantly correlated with the volume of fresh semen (r=-0.30, P=0.04). The breed of boars and the individual boars within the same breed significantly influenced most of the post-thaw sperm parameters.

Keywords: boar, semen cryopreservation, sperm quality, variation

Introduction

Artificial insemination (AI) in pig is nowadays widely used in the pig industry all over the world. Conventionally, the AI was performed using fresh semen with 2 to 5x10^9 motile spermatozoa in 80 to100 mL of volume. AI in sows using frozen-thawed semen has been developed long time ago in many countries in Europe and USA (Larsson and Einarsson, 1976). However, the success of AI using frozen-thawed semen has just markedly progressed during a recent year (Eriksson, 2000). The main reasons may include the development of insemination technique and the advance of knowledge concerning time of ovulation.

The advantages for development of frozen-thawed semen in pig include the preservation of the good genetic resource that can be done for a longer period than the herd life of the boar, the distribution of superior genetic boar that is much quicker than
fresh semen and also the improvement of the transportation of spermatozoa across countries (Polge, 1956; Almlid and Hofmo, 1996; Johnson, 1998). However, boar spermatozoa after being frozen-thawed are weak, and have a lower fertility rate than fresh semen (Eriksson and Rodriguez-Martinez, 2000). The reason is due partly to the lipid structure within the plasma membrane of the boar spermatozoa that is very sensitive to the change in temperature (Johnson et al., 2000).

Frozen-thawed boar sperm quality is influenced by many factors such as freezing and thawing protocols, composition of diluents used in the processes, susceptibility to cryo-injury of the spermatozoa, etc. (Johnson et al., 2000; Suzuki et al., 2005). The susceptibility to cryo-damage of the boar spermatozoa, so called ‘sperm freezability’, is related to the differences of individual boars and the breed of boar (Larsson and Einarsson 1976; Thurston et al., 2002; Holt et al., 2005).

In tropical countries, cryopreservation of boar semen is nowadays performed in a limited scale and it has yet to be conducted in Thailand particularly for the commercial purpose. The present study therefore aimed to established cryopreservation protocol for boar semen in Thailand. Fresh and post-thaw semen quality, sperm concentration and variation among ejaculates were investigated.

**Materials and Methods**

**Animals and Semen**

Fifteen purebred boars (5 Landrace; L, 5 Yorkshire; Y and 5 Duroc; D) from a commercial herd in Nakorn-pathom province, Thailand were used in the experiment. The boars aged between 1 to 3 years old and were being used for routine AI in the herds. The boars were fed approximately 3 kg per day with a corn-soybean-fishmeal base feed containing 15 to16% crude protein. Water was provided ad libitum. Three ejaculates from each boar were collected at one week interval using the gloved-hand technique. The fresh semen used must contain a minimum of 70% sperm motility. The collected semen was sent to the laboratory within 30 min after collection.

**Semen Cryopreservation**

After collection, the semen was diluted with an isothermal Beltsville thawing solution (BTS, Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) extender with a ratio of 1:1 (v/v). Diluted semen was held at 15 °C for 2 h and centrifuged at 800xg for 10 min. The supernatant was discarded and the semen precipitant was re-suspended (about 1 to 2:1) with lactose-egg yolk (LEY) extender (80 mL of 11% lactose solution and 20 mL egg yolk). The diluted semen was cooled down to 5°C within 90 min. Two parts of semen were mixed with one part of extender III consisting of LEY extender with 9% glycerol and 1.5% Equex-STM Paste (Nova Chemical Sales, Scituate Inc., MA, USA) (modified after Westendorf et al., 1975 and Gadea et al., 2004). Thereafter, the processed semen was loaded into 0.5 mL straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before placing in liquid nitrogen (LN2) vapor approximately 3 cm above the level of the LN2 for 20 min and then were plunged into LN2.

**Thawing Process**

Thawing was achieved, approximately one day after storing in LN2, by immersing the straws in water at 50 °C for 12 sec (Selles et al., 2003). After thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The addition of LEY extender was performed to avoid the spermatozoa sticking to the glassware during the sperm motility analysis. The extended thawed semen was incubated in a 38 °C water-bath for 30 min before evaluating the semen quality after thawing.

**Evaluation of Semen Quality**

**Sperm concentration and motility**

Sperm concentration was assessed by direct cell count using Bürker haemocytometer (Boeco, Germany) (Ax et al., 2000). Sperm concentration was expressed as spermatozoa × 10⁶ mL⁻¹. Subjective motility of both fresh and post-thaw spermatozoa was evaluated at 38°C under a light microscope with 400x magnification. The sperm motility examinations for all semen samples were assessed by one person.
Establishment of boar semen cryopreservation in Thailand

Sperm viability and normal apical ridge acrosomes

The percentages of sperm viability and of normal apical ridge (NAR) acrosomes were determined separately by eosin-nigrosin staining (Dott and Foster, 1972). The semen sample (50 µL) was well mixed with a drop of eosin-nigrosin dyes (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland), and the mixture (10 µL) was smeared and dried on a glass slide. Evaluation was undertaken by counting 200 spermatozoa with 1000x magnification. Spermatozoa with an unstained head were regarded as the live spermatozoa. The spermatozoa with a crescent shaped apical ridge were regarded as the NAR spermatozoa.

Functional integrity of sperm plasma membrane

Functional integrity of sperm plasma membrane was assessed using the short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Spermatozoa were incubated at 38 °C for 10 min, with 75 milli-osmole kg⁻¹ a hypo-osmotic solution consisting of 0.368% (w/v) Na-citrate and 0.675% (w/v) fructose (Merck, Germany) in distilled water. Following this incubation time, 200 µL of the semen-hypo-osmotic solution was fixed in 1000 µL of a hypo-osmotic solution plus 5% formaldehyde (Merck, Germany), for later evaluation. Sperm coiling was assessed by placing 20 µL of well-mixed sample on a warm slide, which was covered with a coverslip before being observed under a light microscope (1000x). Two hundred spermatozoa per slide were counted. In order to determine the percentage of spermatozoa with functional membrane intact, the proportion of coiled tail spermatozoa from a control sample (300 milli-osmole kg⁻¹) was subtracted from the result of hypo-osmotic condition.

Sperm plasma membrane integrity

The integrity of sperm plasma membrane was evaluated with SYBR-14/propidium iodide (PI) (Fertilight®; Sperm Viability Kit, Molecular Probes Europe BV, The Netherlands). For staining procedure, 50 µL aliquots of semen (approximately 50×10⁶ spermatozoa mL⁻¹) were mixed with 2.7 µL of the user solution of SYBR-14 and 10 µL of PI. The user solution was SYBR-14 diluted (1:100) in dimethyl sulfoxide (DMSO), fractionated and frozen in eppendorfs. After incubation at 37°C for 20 minutes, 2×100 spermatozoa were assessed (1000x) under a fluorescence microscope. The nuclei of spermatozoa with intact plasma membrane stained green with SYBR-14 while those with damaged membranes stained red with PI. The results were expressed as the percentage of spermatozoa with intact membranes (SYBR).

Statistical Analyses

The statistical analyses were performed using SAS (SAS version 9.0, Cary, N.C., USA). Descriptive statistics was used to describe semen quality before and after thawing. The semen qualities before and after thawing were compared for each boar using paired t-test. Pearson’s correlation was used to evaluate the correlation among all sperm parameters that were measured including the sperm motility, the sperm viability, the NAR, the sHOST and the SYBR. Data on the motility, the viability, the NAR, the sHOST positive spermatozoa and the SYBR after thawing were analyzed using the General Linear Model (GLM) procedure of the SAS. The statistical model used included the effects of the breed of boar (D, L, Y), repeated ejaculation (1 to 3) and the effect of the individual boar nested within breed. Least-square means were obtained from each class of the factors and were compared by using least significant difference test (LSD). The differences with P<0.05 were regarded as statistical significance.

Results

Semen Quality before and after Frozen-Thawed

The semen quality including the sperm motility, the sperm viability, the NAR, the sHOST and the SYBR, of fresh and frozen-thawed semen are presented in Table 1. On average, the sperm concentration of pre-diluted fresh semen was 530×10⁶ spermatozoa mL⁻¹ and the sperm concentration of frozen-thawed semen was 811×10⁶ spermatozoa mL⁻¹, varied between 325×10⁶ and 1620×10⁶ spermatozoa mL⁻¹. The motility of frozen-thawed spermatozoa was 28%, the frozen-thawed sperm viability was 36%, and the proportion of frozen-thawed spermatozoa with intact membrane (SYBR) was 31% (Table 1). All of the sperm parameters significantly decreased after being frozen and thawed. For instance, the sperm motility decreased by 44%, the sperm viability decreased by
38%, and the NAR deceased by 60% ($P<0.001$). It was found that the quality of frozen-thawed semen varied dramatically such as sperm motility varied from 5 to 45%, sperm viability varied from 13 to 60% and SYBR varied from 13 to 53% (Table 1).

**Correlations Among Post-Thaw Sperm Parameters**

Most of the sperm parameters measured after being frozen-thawed were significantly correlated (Table 2). The motility of frozen-thawed spermatozoa was significantly correlated with the sperm viability ($r=0.74; P<0.001$), the NAR ($r=0.33; P=0.03$), the sHOST ($r=0.58; P<0.001$) and the membrane integrity ($r=0.67; P<0.001$) (Table 2). Additionally, it was found that the higher concentration of the frozen-thawed semen resulted in a significantly lower percentage of spermatozoa with intact membranes ($r=-0.3, P=0.04$). Post-thaw sperm concentration was significantly correlated with the volume of fresh semen ($r=-0.30, P=0.04$) (data not shown).

**Factors Influencing Post-Thaw Semen Quality**

It was revealed that the breed of boars and the individual boars within the same breed significantly influenced most of the frozen-thawed sperm parameters (Table 3 and Figure 1). For instance, the post-thaw sperm viability in D and L boars was significantly higher than Y boars ($P<0.05$). The motility and the NAR of frozen-thawed spermatozoa were lowest in Y boars. The membrane integrity (SYBR) of frozen-thawed spermatozoa in the L boars was significantly higher than the Y boars ($P<0.05$; Figure 1). Y boars had the highest variation of the plasma membrane integrity of the spermatozoa after being frozen-thawed compared to other breeds (data not shown). Repeated ejaculation significantly influenced the percentage of frozen-thawed spermatozoa with intact membranes ($P<0.01$; Table 3). On average, the last ejaculate had the highest percentage of spermatozoa with intact membranes (29, 29 and 35% for ejaculation 1, 2 and 3, respectively; data not shown). However, repeated ejaculation within boar did not influence other post-thaw sperm parameters ($P>0.1$).

**Discussion**

The present study demonstrated that cryopreservation of boar semen could be successfully established in Thailand. However, a considerably high variation of post-thaw semen quality was observed. In general, successful boar semen cryopreservation depends on a number of factors, which could be classified as internal factors and external factors. The internal factors include the inherent characteristic of spermatozoa, and differences between boar and ejaculate. The external factors include the composition of diluents, Table 1 Descriptive statistics for sperm parameters measurements of fresh ($n=45$) and frozen-thawed semen ($n=45$) from 15 boars.

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Fresh semen</th>
<th>Frozen-thawed semen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Concentration ($\times 10^6$ sperm mL$^{-1}$)</td>
<td>530</td>
<td>175-2125</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>72</td>
<td>70-80</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>74</td>
<td>57-87</td>
</tr>
<tr>
<td>NAR (%) $^1$</td>
<td>86</td>
<td>67-98</td>
</tr>
<tr>
<td>sHOST (%) $^2$</td>
<td>54</td>
<td>21-89</td>
</tr>
<tr>
<td>SYBR (%) $^3$ $^4$</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^1$ NAR = normal apical ridge  
$^2$ sHOST = functional integrity of sperm plasma membrane  
$^3$ SYBR = percentage of spermatozoa with intact membranes  
$^4$ NA = not available
Table 2 Pearson’s correlation coefficient (r) and significance level (P-value) among sperm parameters measurements after frozen-thawed\textsuperscript{1}.

<table>
<thead>
<tr>
<th></th>
<th>Viability (%)</th>
<th>NAR (%)\textsuperscript{2}</th>
<th>sHOST (%)\textsuperscript{2}</th>
<th>SYBR (%)\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm motility (%)</td>
<td>0.74***</td>
<td>0.33*</td>
<td>0.58***</td>
<td>0.67***</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>0.50***</td>
<td>0.72***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAR (%)</td>
<td>0.18 ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sHOST (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.49***</td>
</tr>
</tbody>
</table>

\textsuperscript{1} * = P≤0.05, ** = P≤0.01, *** = P≤0.001, ns = P>0.05,
\textsuperscript{2} NAR = normal apical ridge
\textsuperscript{3} sHOST = functional integrity of sperm plasma membrane
\textsuperscript{4} SYBR = percentage of spermatozoa with intact membranes

Table 3 Significance levels of factors affecting post-thaw semen quality\textsuperscript{1,2}.

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>NAR (%)</th>
<th>sHOST (%)</th>
<th>SYBR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed</td>
<td>2</td>
<td>0.14</td>
<td>0.01</td>
<td>0.11</td>
<td>0.87</td>
<td>0.05</td>
</tr>
<tr>
<td>Ejaculate</td>
<td>2</td>
<td>0.23</td>
<td>0.22</td>
<td>0.46</td>
<td>0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>Boar\textsuperscript{3}</td>
<td>12</td>
<td>0.04</td>
<td>0.04</td>
<td>0.03</td>
<td>0.002</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{1} All factors were included in the same statistical model
\textsuperscript{2} P-value were calculated based on type III sum of square
\textsuperscript{3} Effect of individual boars was nested within breed

Figure 1 Semen quality after frozen-thawed in Duroc, Landrace and Yorkshire boars, different letter within each sperm parameter differ significantly (P<0.05) (NAR= normal apical ridge, sHOST= functional integrity of sperm plasma membrane, SYBR = percentage of spermatozoa with intact membranes).

The present study demonstrated a significant impact of internal factors, i.e. the breed of boar and individual boars, on the post-thaw semen quality. The individual boars influenced the sperm susceptibility to cryoinjury as shown that the post-thaw sperm quality differed significantly among boars within the same breed. This was in agreement with previous findings by Holt et al. (2005) and Larsson and Einarsson (1976). Watson (1996) suggested that cold shock resistance of spermatozoa may relate to the lipid composition within the membrane bilayer of the sperm plasma membrane. Generally, boar spermatozoa which have a high sensitivity to cold shock contain a low percentage of phosphatidylcholine and a high percentage of phosphatidylethanolamine and sphingomyelin in their plasma membrane (Johnsson et al., 2000). The proportion of cholesterol in the sperm plasma membrane also influences the thermotrophic behavior of spermatozoa. In boar spermatozoa, cholesterol/phospholipids ratio is low as well as the cholesterol is distributed asymmetrically and is presented in the outer than the inner layer of the membrane. These render the type and concentration of cryoprotective agents, rates of dilution and of cooling, equilibration and method of freezing and thawing of semen (Johnson et al., 2000).
inner layer of the membrane is vulnerable to cold shock. The difference in physiological characteristic of the spermatozoa from each boar may contribute to the individual variation. Further studies concerning the fat composition of the boar spermatozoa are of interest.

In the present study, Y boars seemed to have the most variations in many of the frozen-thawed sperm parameters. This finding was in agreement with the results of Thurston et al. (2002) who found that the Y breed was more evenly split of sperm freezability than the others. The reason for this is not known. Additional studies with a higher number of boars within each breed need to be performed. It has been demonstrated that there is a correlation between some genetic markers and the differences in the sensitivity of boar spermatozoa to cryoinjury (Thurston et al., 2002; Holt et al., 2005). Holt et al. (2005) conducted an experiment with more than 100 boars and use the genetic analyses to test that the susceptibility of boar spermatozoa to cryo-injury is a consistent feature of individual and found that those individual differed in susceptibility. The genomic differences between individual boars correlated with freezability and post-thaw quality of their spermatozoa. Thurston et al. (2002) analyzed genome of 22 boars by amplified restriction fragment length polymorphism and found that 16 candidate molecular markers linked to genes controlling semen freezability. These genomes varied among individual boars, and may lead to an improvement in the predictability of both post-thaw semen quality and fertility of individual males.

The number of spermatozoa loaded in each semen package also influences the frozen-thawed sperm quality. In this study, higher concentration of frozen-thawed semen resulted in the lower membrane integrity. The breed of boars and the individual boars within the same breed significantly influenced most of the post-thaw sperm parameters.

Conclusions

Boar semen cryopreservation could be successfully performed in Thailand with the averages of 811×10^6 mL⁻¹ frozen-thawed sperm concentration, of 28% the sperm motility and of 36% the sperm viability. However, post-thaw sperm concentration and semen quality varied considerably. The higher concentration of frozen-thawed semen resulted in the lower membrane integrity. The breed of boars and the individual boars within the same breed significantly influenced most of the post-thaw sperm parameters.

Acknowledgments

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