**Research Report**

# DEVELOPMENT OF TECHNIQUES FOR FREEZING BOAR SEMEN


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**April 2006**

**1. Research title: Development of techniques for freezing boar semen**

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**4. Abstract/ Research summary**

The objective of the present study was to investigate the feasibility of boar semen cryopreservation in Thailand and to investigate factors that could affect the boar semen quality after cryopreservation. Fifteen purebred boars (5 Landrace, 5 Yorkshire and 5 Duroc) from 2 commercial herds in Nakorn-prathom province were used in the experiment. Three ejaculates from each boar were collected with over one-week interval using gloved-hand technique. After collection, the semen was diluted with isothermal Beltsville thawing solution (BTS) extender. Diluted semen was held at 15 °C for 2 h and centrifuged. The supernatant was discarded and the semen precipitant was re-suspended with lactose-egg yolk (LEY) extender. The diluted semen was cool down to 5°C within 90 min. Two parts of semen were mixed with one part of LEY extender with 9% glycerol and 1.5% Equex-STM®. Thereafter, the processed semen was loaded into 0.5 mL straws. The straws were placed in liquid nitrogen ($LN_2$) vapor approximately 3 cm above the level of the $LN_2$ for 20 min and then were plunged into $LN_2$. Thawing was achieved by immersing the straws in water at 50 °C for 12 second. After thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The extended thawed semen was incubated in a 38 °C water-bath for 30 min before evaluating the semen quality after thawing. Sperm concentration, individual motility, sperm viability, percentage of normal apical ridge (NAR), sperm plasma membrane function (sHost) and sperm plasma membrane integrity (SYBR) were evaluated. 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. Data on individual motility, viability, NAR, sHost positive spermatozoa and SYBR after thawing were analyzed using the General Linear Model (GLM) procedure of the SAS. On average, the sperm concentration of pre-diluted fresh semen was $529.7x10^6$ spz/mL and the sperm concentration of frozen thawed semen was $811x10^6$ spz/mL. The individual motility, the sperm viability, the NAR, the sHost and the SYBR of boar spermatozoa after frozen-thawed were 28%, 36.2%, 26.4%, 18.5% and 30.9%, respectively. All of the sperms parameters measured significantly decreased after frozen and thawed i.e., individual motility decreased by 44.2%, sperm viability decreased by 37.9% and NAR deceased by 59.5%. The individual motility of frozen-thawed spermatozoa was 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, $P=0.04$). Breed of boars and the individual boar within the same breed significantly influenced most of the sperm parameters after being frozen-thawed. The sperm viability of frozen-thawed semen in Duroc and Landrace boars was significantly higher than Yorkshire boars ($P<0.05$). The membrane integrity of frozen-thawed semen in Landrace boar was significantly higher than Yorkshire boars ($P<0.05$). In conclusions, the semen cryopreservation of boar could be performed successfully in Thailand with an average concentration of spermatozoa in frozen thawed semen of $811x10^6$ spz/mL, the individual motility of 28% and the sperm viability of 36%. Breed of boars and the individual boar within the same breed significantly influenced most of the sperm parameters after being frozen-thawed.

*Keywords:* pig, reproduction, artificial insemination, frozen semen

## 5. Research background

Artificial insemination (AI) in pig is nowadays widely used in the pig industry all over the world. In USA, over 50% of the pig production is produced by AI (Johnson et al., 2000). AI in some country in Europe and some herd in Thailand is nowadays over 90% (Tummaruk et al. 2000, 2004). Conventionally, the AI was performed using fresh semen with 2-5x$10^9$ motile spermatozoa in 80-100 mL of volume. The semen is inseminated to the sow intra-cervically for 2-4 times during the standing oestrus. 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 markedly progress 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 advantage for development of frozen semen in pig included the preservation of the good genetic resource can be done for a longer period than the herd life of the boar, the distribution of superior genetic boar are 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 frozen-thawed are weak, and had a lower fertility rate than fresh semen (Eriksson and Rodriguez-Martinez, 2000). The reason are due to the lipid structure within the plasma membrane of the boar spermatozoa are very sensitive to the change in temperature (Johnson et al., 2000).

Boar semen differs in several aspects from the semen of other domestic animals. It is produced in large volumes and is extremely vulnerable to cold shock. These and other characteristics of boar semen require special consideration in the design of freezing protocols (Johnson et al., 2000). Important factors to be considered for freezing of boar semen included composition of diluents, type and concentration of cryo-protective agent, rates of dilution and cooling, equilibration and methods of freezing and thawing of semen. For instance, egg yolk, which is known as a cryoprotective agent for spermatozoa of various domestic animals, is not give the same level of protection to the boar spermatozoa (Benson et al., 1967). However, its protective effect can be improved by adding Orvus Es Paste (OEP) to the extender (Graham et al., 1971; Pursel et al., 1978). OEP, nowadays known as Equex-STM ®, a synthetic detergent based on sodium and triethanolamine lauryl sulphate, have been included in most egg yolk-containing-diluents for freezing of boar semen (Johnson et al., 2000).

Since AI in pig need a lot of spermatozoa per dose, the container of frozen thawed semen become a problem for the development of commercialized frozen-thawed semen (Bwanga et al., 1991). In general, large container for high semen volume such as maxi straw (5 mL) resulted in sub-optimal motility after thawing due to the difficulty of temperature control within the straw during freezing process. Small container such as mini straw (0.25 and 0.5 mL) resulted in good semen quality after thawing but low number of spermatozoa could be load in one straw, which is not enough for a single dose of insemination (about 250-500 x $10^6$ total number of spermatozoa per straw). The packaging of frozen semen as a pellet was a convenient solution but the labeling and the high risk of contamination have been concerned. Theoretically, the packages with larger surface-to-volume ratio, e.g., pellets, 0.25 mL straws, allow more homogeneous freezing and thawing throughout the sample. Thus frozen thawed spermatozoa using these packages are less damaged than those in the smaller surface-to-volume ratio packages, e.g., 5 mL straws (Eriksson, 2000). Due to the limitation of freezing package, studies in the area of frozen thawed semen's container are still going on. Recently, Eriksson and Martinez (2000) found that Flatpack® could be used as an option for a container for large semen volume (5 mL, 5,000x$10^6$ total number of spermatozoa per straw). The sperm quality and the farrowing rate after AI with frozen semen in Flatpack® container were better than maxi-straw (Eriksson and Rodriguez-Martinez, 2000).

Freezing and thawing rate of semen have a large impact on the survival of spermatozoa after thawing. The slow freezing rate resulted in the formation of ice crystal within the sperm cell and therefore, the semen may need a high concentration of cryopreservative agent such as glycerol, egg yolk, propylene glycol, EDTA and sodium dodecyl sulphate (SDS). It has been demonstrated that adding a detergent sodium dodecyl sulphate to freezing extenders improved the post-thaw survival and longevity of spermatozoa in many species including boar spermatozoa (Graham et al., 1971; Westendorf et al., 1975).

Many freezing rate and various proportion of cryopreservative agent within the diluents have been demonstrated to be effective such as freezing rate 30 °C/min with diluents contained 3% glycerol in 0.50 mL straw (Fiser and Fairfull, 1990), freezing rate 50 °C/min with diluents contained 1.5% glycerol in 0.25 mL straw (Woelders and Den Besten, 1993). In general, it was suggested that the large straw need a slower freezing rate and a higher proportion of glycerol than the small straw (Pursel and Park, 1985).

For boar semen cryopreservation, holding time has been proved to be a very important factor for the post-thawed semen quality. Eriksson (2000) suggested that boar semen should be leave at 15 °C for 1-5 h before decreasing the temperature below 15 °C to avoid cold shock.

In general, the frozen thawed spermatozoa are weak and the freezing in small volume, in most case, resulted in a better post-thawed semen quality than freezing in a large volume. It is well established that, using the conventional AI technique, the spermatozoa were transported from the cervix of the female through the uterine horns until reaching the utero-tubal junction and the oviduct, where the spermatozoa can be deposited and wait for ovulation. These transport resulted in the loss of more than 90% of the inseminated spermatozoa via semen backflow and phagocytosis within the uterus (Mburu et al., 1996; Stervink et al., 1998). Development of insemination technique with a low number of spermatozoa in pig has therefore being investigated (Kruger et al., 1999; Martinez et al., 2001, 2002; Vazquez et al., 2003, 2005).

Frozen thawed spermatozoa could be survived for a shorter period than spermatozoa from the diluted fresh semen. The reason is due to the freezing and thawing process induced sperm capacitation (Eriksson and Rodriguez-Martinez, 2000; Eriksson et al., 2002).

Recently, deep intra uterine insemination (DIUI) using a special designed catheter, length 180 cm outer diameter 4 mm and working channel 1.8 mm, has been developed (Martinez et al., 2001; Vazquez et al., 2005). The catheter could be inserted through the uterine horn and deposited semen in one horn at the proximal third of the uterine horn, closed to the sperm reservoir. Earlier studies have shown that the flexible catheter could be passed through the cervix completely in 90-95% of multiparous sows (parities 2-6; n=147) within about 4 min/insemination (Martinez et al. 2001, 2002). Using this technique a 20-fold reduction in the number of spermatozoa could be used without any significant effect on farrowing rate (FR) and litter size (Martinez et al., 2002). Martinez et al. (2002) demonstrated that FR and number of total piglets born/litter (TB) after DIUI with $150x10^6$ spermatozoa/dose in 5 mL volume compared to conventional AI with 3,000 x $10^6$ spermatozoa/dose in 100 mL volume did not differ significantly (FR 82.9 versus 83% and TB 9.7 versus 9.9 piglets/litter, respectively).

DIUI technique have also been applied for some new sperm technology e.g., frozen-thaw semen and sex sorted sperm and maximizing the used superior boar sperm by using a low sperm number per dose of insemination (Roca et al., 2003; Vazquez et al., 2005). Roca et al. (2003) inseminated 49 hormonally treated weaned sows once with frozen-thawed sperm $1000x10^6$ spermatozoa/dose in 5 mL using DIUI technique resulted in 77.5% FR and 9.3 piglets/litter, which was not differ significantly from frozen-thawed sperm $6000x10^6$ spermatozoa/dose in 100 mL inseminated using conventional AI technique (75.8% FR and 9.6 piglets/litter, n=33). In normal weaned sows inseminated twice at 30 and 42 h after onset of oestrus with DIUI technique during oestrus, frozen-thaw semen ($1,000x10^6$ spermatozoa/dose) resulted in a lower FR than fresh semen ($150x10^6$ spermatozoa/dose) (70.0 versus 84.2%). Vazquez et al. (2003) demonstrated that hormonally treated weaned sows (n=45) inseminated once at 38 h after HCG treatment with $140x10^6$ flowcytometry sorted spermatozoa using DIUI technique resulted in 46.6% FR and 8.2 piglets/litter, while the sows inseminated with fresh semen (n=49) using the same technique and same number of spermatozoa resulted in 80.9% and 9.5 piglets/litter.

DIUI have been performed successfully in Thailand using diluted fresh semen (Tummaruk et al., 2005). The DIUI with frozen thawed semen should be therefore investigated further. However, the boar semen freezing technique has never been developed in Thailand.

## 6. Objectives

The objectives of the experiment were:

1. To study the feasibility of boar semen cryopreservation in Thailand
2. To study factors affecting the boar semen quality after cryopreservation

## 7. Research methodology

Experimental research

## 8. Study period

1st October 2004- 30th September 2005

## 9. Materials and methods

*Animals*

Fifteen purebred boars (5 Landrace, 5 Yorkshire and 5 Duroc) from 2 commercial herds (A and B) in Nakorn-prathom province, Thailand were used in the experiment. The boar age between 1-3 years old and are being used for routine artificial insemination (AI) in the herds. In herd A (n=8; 5 Duroc, 1 Landrace and 2 Yorkshire), the boars were allocated in an evaporative cooling system and in herd B (n=7; 4 Landrace and 3 Yorkshire), the boars were allocated in a conventional open house system. The boars were fed approximately 3 kg per day (twice a day) with a corn-soybean-fishmeal base feed containing protein about 15-16%. Water was provided ad libitum. Routinely, the Duroc boars were used for semen collection once weekly, while semen collection was performed in L and Y boars in a longer interval (about 2-3 weeks interval).

*Semen collection*

Three ejaculates from each boar were collected with over one-week interval using gloved-hand technique. The fresh semen used had to have a minimum of 70% individual motility and 80% morphologically normal spermatozoa. The collected semen was sent to the laboratory within 30 minute after collection.

*Semen freezing protocols*

After collection, the semen was diluted with 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 later centrifuged at 800xg for 10 min. The supernatant was discarded and the semen precipitant was re-suspended (about 1-2:1) with lactose-egg yolk (LEY) extender (80 mL of 11% lactose solution and 20 mL egg yolk). The diluted semen was cool down to 5°C within 90 min. Two parts of semen were mixed with one part of LEY extender with 9% glycerol and 1.5% Equex-STM® (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 final concentration of semen to be frozen was approximately $1,000x10^6$ spermatozoa/mL and contained 3% glycerol. The straws were sealed with PVC powder before placing in liquid nitrogen ($LN_2$) vapor approximately 3 cm above the level of the $LN_2$ for 20 min and then were plunged into $LN_2$.

*Semen thawing protocol*

Thawing was achieved, approximately 20-30 min after storing in $LN_2$, by immersing the straws in water at 50 °C for 12 second (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 stick to the glassware during the individual motility analysis. The extended thawed semen was incubated in a 38 °C water-bath for 30 min before evaluating the semen quality after thawing.

*Semen evaluation*

*Sperm concentration and individual motility*

Sperm concentration was assessed by direct cell count using Burker haemocytometer (Boeco, Germany) (Beardon and Fuquay, 1997). Sperm concentration was expressed as spermatozoa $x10^6$ /mL. Subjective motility of both fresh and post thawed spermatozoa was evaluated at 38 °C under light microscope with 400x magnification. The individual motility examinations for all semen samples were assessed by one person.

*Sperm viability and normal apical ridge*

The percentages of sperm viability and normal apical ridge (NAR) were determined by eosin-nigrosin staining (Dott and Foster, 1972; Beardon and Fuquay, 1997). The semen sample (50 μL) were 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 live spermatozoa. Spermatozoa with a crescent shaped apical ridge were regarded as normal apical ridge (NAR) spermatozoa.

*Functional integrity of sperm plasma membrane*

Functional integrity of sperm plasma membrane was assessed using a short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Spermatozoa were incubated, at 38 °C for 10 min, with 75 mOsm/kg a hypo-osmotic solution that consisted 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. Coiled tail (sHOST positive) spermatozoa found following incubation were functional intact plasma membrane.

*Sperm 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). 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, 2x100 spermatozoa were assessed (x1000) under 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 analysis*

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, i.e, individual motility, sperm viability, NAR, sHost and SYBR. Data on individual motility, viability, NAR, sHost positive spermatozoa and SYBR after thawing were analyzed using the General Linear Model (GLM) procedure of the SAS. The statistical model used included the effects of breed of boar (Duroc, Landrace and Yorkshire), repeated ejaculation (1 to 3) and effect of individual boar nested within breed. Least-square means were obtained from each class of the factors and were compared by using least significant different test (LSD). The differences with $P<0.05$ was regarded as statistical significance.

## 10. Results

*Semen quality before and after frozen-thawed*

The semen quality, as indicated by the individual motility of spermatozoa, the sperm viability, NAR, sHost and SYBR, of fresh and frozen-thawed are presented in Table 1. On average, the sperm concentration of the pre-diluted fresh semen was $529.7x10^6$ spermatozoa/mL and the sperm concentration of the frozen-thawed semen was $811x10^6$ spermatozoa/mL. The individual motility of frozen-thawed semen was 28.0%, the sperm viability was 36.2% and the proportion of spermatozoa with intact membrane after frozen-thawed (SYBR) was 30.9% (Table 1). All of the sperms parameters measured significantly decreased after being frozen and thawed (Table 2). For instance, the individual motility decreased by 44.2%, the sperm viability decreased by 37.9% and NAR deceased by 59.5% (Table 2). It was found that the quality of frozen-thawed semen varied dramatically, for instance, individual motility varies from 5 to 45%, sperm viability varied from 13-60% and SYBR varied from 13-53% (Table 1).

**Table 1** Descriptive statistics for sperm parameters measurements of fresh (n=45) and frozen-thawed semen (n=45) from 15 boars (5 Landrace, 5 Yorkshire and 5 Duroc)

| Sperm parameters | Fresh semen | | Frozen-thawed semen | |
|---|---|---|---|---|
| | mean | range | mean | range |
| Concentration ($x10^6$ spz/ml) | 529.7 | 175-2125 | 811.0 | 325-1620 |
| Individual motility (%) | 72.2 | 70-80 | 28.0 | 5-45 |
| Sperm viability (%) | 74.2 | 57-87 | 36.2 | 13-60 |
| NAR (%)[1] | 85.9 | 67-98 | 26.4 | 8-55 |
| sHost (%)[2] | 54.1 | 21-89 | 18.5 | 3-45 |
| SYBR (%)[3] | NA[4] | NA | 30.9 | 13-53 |

[1]NAR= normal apical ridge, [2]sHost = functional integrity of sperm plasma membrane, [3]SYBR = percentage of spermatozoa with intact membranes, [4]NA = did not measure

**Table 2** Comparisons of semen quality before and after frozen-thawed

| Sperm parameters | Before | After | Difference [4] | *P*-value [5] |
|---|---|---|---|---|
| Individual motility (%) | 72.2±3.1 | 28.0±9.3 | -44.2 | <0.001 |
| Sperm viability (%) [1] | 74.2±6.4 | 36.2±11.2 | -37.9 | <0.001 |
| NAR (%) [2] | 85.9±6.7 | 26.4±9.4 | -59.5 | <0.001 |
| sHost (%) [3] | 54.1±15.9 | 18.5±11.0 | -35.6 | <0.001 |

[1]NAR= normal apical ridge, [2]sHost = functional integrity of sperm plasma membrane, [3]SYBR = percentage of spermatozoa with intact membranes, [4] The average of reduction from each ejaculation, [5] Paired *t*-test

*Correlations among sperm parameters after frozen-thawed*

Most of the sperm parameters measured after frozen-thawed were significantly correlated (Table 3). The individual motility of frozen-thawed spermatozoa was significantly correlated with sperm viability (r=0.74; *P*<0.001), NAR (r=0.33; *P*<0.02), sHOST (r= 0.58; *P*<0.001) and membrane integrity (r=0.67; *P*<0.001) (Table 3). Additionally, it was found that the higher concentration of frozen-thawed semen resulted in a significantly lower percentage of spermatozoa with intact membranes (r=-0.3, *P*=0.04).

**Table 3** Pearson's correlation coefficient (r) and significance level (*P*-value) among sperm parameters measurements after frozen-thawed

| | Viability (%) | NAR (%) | sHost (%) | SYBR (%) |
|---|---|---|---|---|
| Individual motility (%) | 0.74*** | 0.33* | 0.58*** | 0.67*** |
| Sperm viability (%)[1] | | 0.50*** | 0.72*** | 0.54*** |
| NAR (%)[2] | | | 0.18 ns | 0.11 ns |
| sHost (%)[3] | | | | 0.49*** |

* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, ns = $P > 0.05$, [1]NAR= normal apical ridge, [2]sHost = functional integrity of sperm plasma membrane, [3]SYBR = percentage of spermatozoa with intact membranes

*Factors influencing the quality of spermatozoa after frozen-thawed*

It was revealed that breed of boars (Duroc, Landrace and Yorkshire) and the individual boar within the same breed significantly influenced most of the sperm parameters after frozen-thawed (Table 4). For instance, the sperm viability of frozen-thawed semen in Duroc and Landrace boars was significantly higher than Yorkshire boars ($P<0.05$) (Fig. 1). The individual motility and the NAR of frozen-thawed semen were lowest in Yorkshire boar (Fig. 1). The membrane integrity of frozen-thawed semen in Landrace boar was significantly higher than Yorkshire boars ($P<0.05$) (Fig. 1). The influences of the individual boar on the sperm motility and the plasma membrane integrity of spermatozoa are presented in Fig 2 and Fig. 3, respectively. Fig. 3 demonstrated that Yorkshire boars seem to have a rather high variation of the plasma membrane integrity of spermatozoa after being frozen-thawed.

**Table 4** Significance levels of factors affecting semen quality after frozen-thawed

| Factors [1] | DF[2] | Motility (%) | Viability (%) | NAR (%) | sHost (%) | SYBR (%) |
|---|---|---|---|---|---|---|
| Breed | 2 | 0.14[3] | 0.01 | 0.11 | 0.87 | 0.05 |
| Ejaculate | 2 | 0.23 | 0.22 | 0.46 | 0.39 | 0.01 |
| Boar [4] | 12 | 0.04 | 0.04 | 0.03 | 0.002 | 0.05 |

[1]All factors were included in the same statistical model, [2]DF= degree of freedom, [3]*P*-value were calculated base on type III sum of square, [4]Effect of individual boar was nested within breed

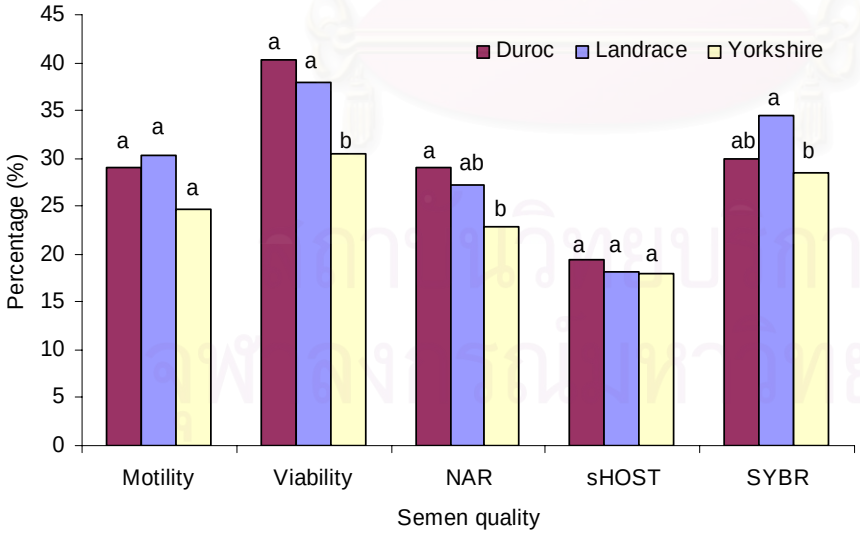


*Fig. 1* Semen quality after frozen-thawed in Duroc, Landrace and Yorkshire boars, different letter within each sperm parameter differ significantly ($P<0.05$) (NAR= normal apical ridge, sHost = functional integrity of sperm plasma membrane, SYBR = percentage of spermatozoa with intact membranes)

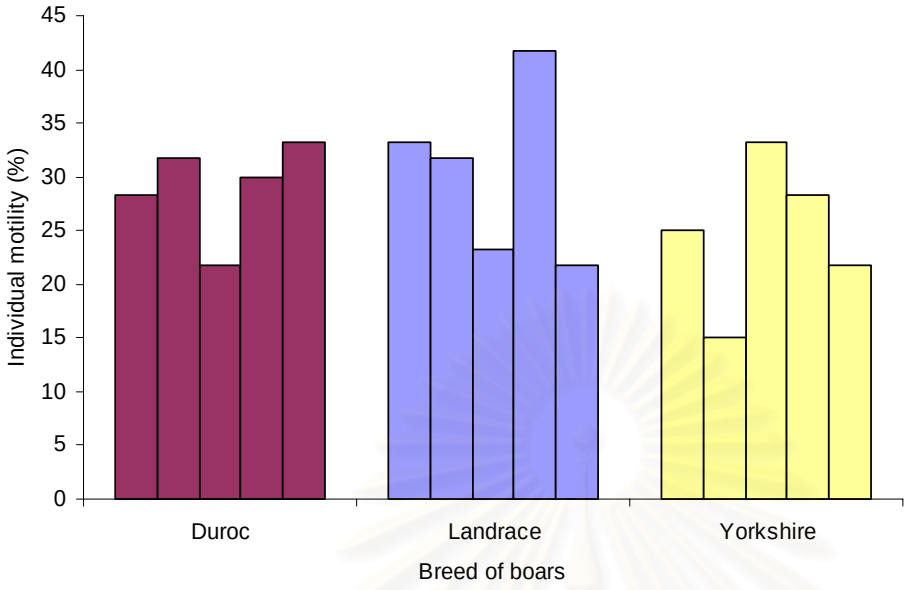


*Fig. 2* Individual motility (%) of frozen-thawed semen in each individual of Duroc, Landrace and Yorkshire boars

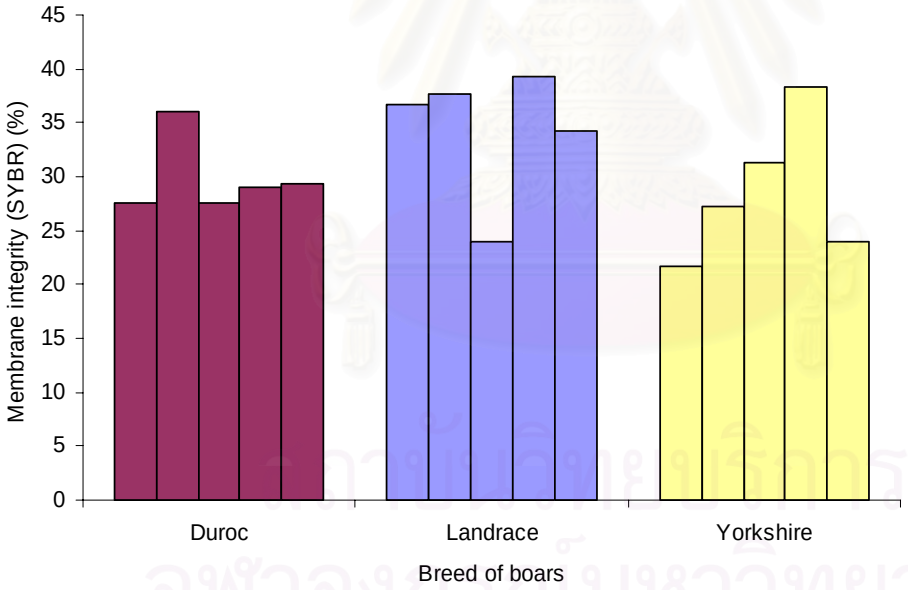


*Fig. 3* Membrane integrity (SYBR) (%) of frozen-thawed semen in each individual of Duroc, Landrace and Yorkshire boars

**11. Discussion & Implementation**

Frozen boar semen has been available commercially since 1975 both in pellet and in straws. However, less than 1% of all insemination are made using frozen thawed semen, most often after export from one country to another and primarily for the purpose of upgrading the genetic base in a particular country or herd (Johnson et al., 2000). The present study demonstrated that boar semen cryopreservation

could be established in Thailand. However, a large variation on the semen quality after being cryopreserved was found. Further study need to be performed to improve semen quality after being frozen and thawed. Earlier studies suggested that 2 main factors that influence sperm cell function after ejaculation and during in vitro storage included the temperature and the suspension medium (Johnson et al., 2000).

Successful freezing of boar semen depends on a number of factors, which could be classified as internal factors and external factors. The internal factors included the inherent characteristic of spermatozoa, and difference between boar and ejaculates. The external factors included the composition of diluents, 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). The present study demonstrated a significant impact of internal factors i.e., breed of boar and individual boar effect on the post-thawed semen quality. It was found that Yorkshire boars seem to have the most variations in many of the post-thawed sperm parameters. The reason for this is not yet known. In the present study, ejaculations seem to have a minor influence on the post-thawed sperm parameter compared to the boar and the breed effect. The significant impact of ejaculation was only found on the membrane integrity.

It is known that boar spermatozoa are very susceptible to cold shock. This occurs when freshly ejaculated boar spermatozoa are cool quickly from body temperature to temperature below 15 °C, which resulted in a loss of viability. However, when fresh semen is held above 15 °C for several hours, the spermatozoa acquire a gradual resistance to cold shock (Pursel et al., 1973). In the present study, the holding time and holding temperature was 2 h and 15 °C. Earlier study demonstrated that pre-incubation for 24 h before a decrease of temperature below 15 °C enhances cold shock resistance (reviewed by Johnson et al., 2000). Watson (1996) suggested that cold shock may be related to the lipid composition within the membrane bilayer of the sperm plasma membrane. In general, bull spermatozoa are more resistance to the cold shock and also to the freezing and thawing process than the boar spermatozoa. The difference between bull (cold resistant) and boar (cold sensitive) spermatozoa could be explained by the difference in the membrane composition. The most difference in the type of phospholipids between boar and bull spermatozoa is that boar spermatozoa contained a low percentage of phosphatidylcholine and a high percentage of phosphatidyethanolamine and sphingomyelin (Johnsson et al., 2000). In addition, the proportion of cholesterol in the sperm plasma membrane influences the thermotrophic behaviour of spermatozoa. In boar spermatozoa, cholesterol/phospholipids ratio is low and the cholesterol is distributed asymmetrically and is presented in the outer than the inner layer of the plasma membrane. This resulted in the inner layer of the membrane are vulnerable to cold shock. Cold could induce reorganization of membrane particles, therefore the function of sperm plasma membrane could be changed in a number of ways. For instance, an increase of permeability, a reduction in enzyme activity and change in lateral motion in channels (De Leeuw et al., 1990).

Dilution and cooling make the boar sperm membrane more permeable (Ortman and Rodriguez-Martinez, 1994). This could stimulate calcium dependent process associated with capacitation and consequently shorten the mechanism of capacitation process. In practice, the frozen-thaw boar semen must be inseminated within 6 hours prior to ovulation to obtain an acceptable fertility (Eriksson, 2000). These indicated that to obtain an acceptable level of fertility rate for boar semen cryopreservation, not only the improvement of post thawed sperm viability but also the development of optimal AI technique for frozen-thawed boar semen. Since the DIUI technique have been established in our laboratory, the implement of DIUI for frozen-thawed semen are therefore being progress (Buranaamnuay et al., 2006b).

The semen diluents are also significantly influence sperm function. The important factors to be considered included the pH, ionic strength, type of ions and osmotic pressure of the medium. Anti-microbial substance often included in the diluents. The optimal pH for boar semen is 7.2-7.5. Boar spermatozoa tolerate a relatively wide rage of osmolality between 240-380 mosM. Ethylenediamine-tetra-acetic acid (EDTA), a BTS compound, captures Ca++ and limits the ion movement across plasma membrane (Watson, 1990). This prevents the initiation of capacitation and the acrosome reaction. The inclusion of EDTA in the diluents of boar semen is therefore of important. In addition, a cryo-protective agent, Equex-STM®, was introduced to the present study. Our preliminary result found that the addition of

Equex-STM® to the freezing diluents significantly enhanced sperm viability, membrane integrity as well as other sperm parameter measured (Buranaamnuay et al., 2006a). Protective effect of Equex-STM® is an indirect effect, by dissolving or dispersing protective components in the egg yolk of the freezing extender. This increases the protective effect of the egg yolk on the sperm membrane stability, rather than by directly affecting cellular membranes (Pursel et al., 1978; Strzezek et al., 1984). The addition of Equex-STM® into an extender without egg yolk exerted a detrimental effect on boar sperm quality (Pursel et al., 1978).

In conclusions, the semen cryopreservation in boar could be performed successfully in Thailand with an average concentration of spermatozoa in frozen thawed semen of $811x10^6$ spz/mL, the individual motility of 28% and the sperm viability of 36%. Breed of boars and the individual boar within the same breed significantly influenced most of the sperm parameters after being frozen-thawed.

**Acknowledgements**

This study was granted by the Research and Development Center for Livestock Production Technology, Chulalongkorn University. Miss Kakanang Buranaamnuay is a PhD student under the Royal Golden Jubilee (RGJ) scholarship of Thailand Research Fund (TRF).

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**13. Research output**

1. Buranaamnuay, K., Singlor, J., Tummaruk, P. and Techakumphu, M. 2006a. The success of boar semen cryopreservation in Thailand: impacts of straw volume and Equex-STM®. Proc 19th IPVS Congress, 16-19 July 2006, Copenhagen, Denmark. (accepted)
2. Buranaamnuay, K., Wongtawan, T., Kaeoket, K., Tummaruk, P. and Techakumphu, M. 2006b. Birth of piglets after intra-uterine artificial insemination with frozen-thawed boar semen in Thailand. Proc 19th IPVS Congress, 16-19 July 2006, Copenhagen, Denmark. (accepted)

# THE SUCCESS OF BOAR SEMEN CRYOPRESERVATION IN THAILAND: IMPACTS OF STRAW VOLUME AND EQUEX-STM®

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## Introduction

Frozen-thawed (FT) boar semen quality are affected by factors, such as type of freezing package (1) and a detergent sodium dodecyl sulphate (SDS) in freezing extenders (2). The aims of the present study were to investigate the effect of straw volume (0.25 vs 0.5 mL) and Equex-STM® (Nova Chemical Sales, Scituate Inc., MA, USA), a commercial preparation of SDS added to a freezing extender, on boar sperm quality after cryopreservation.

## Materials and methods

Three ejaculates of semen from each of 4 boars (3 Landrace and 1 Yorkshire) were collected and frozen. Semen was diluted with isothermal Beltsville thawing solution (BTS) extender, placed at 15°C for 2 h and centrifuged at 800xg for 10 min. The semen precipitant was re-suspended (1:1) with lactose-egg yolk (LEY) extender (80 mL of 11% lactose solution and 20 mL egg yolk). After further cooling to 5°C for 90 min, semen were mixed with LEY extender and 9% glycerol with or without 1.5% Equex-STM® (2:1). Experiment I, the processed semen (with 1.5% Equex-STM®) was loaded into 0.25 mL and 0.5 mL straws. Experiment II, the semen containing Equex-STM® and the Equex-free semen were only packaged in 0.5 mL straws. Semen-filled straws were placed in liquid nitrogen ($LN_2$) vapor for 20 min and plunged into $LN_2$ until thawing. Thawing was achieved by immersing the straws in water at 50°C for 12 sec. The thawed semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender and incubated at 38°C for 30 min before evaluating the sperm quality. Data on sperm quality were analyzed by general linear model procedure (GLM) using SAS.

## Results and Discussion

In the present study, weeks of collection did not influence FT sperm quality. Boar significantly affected ($P<0.05$) FT sperm quality. It was revealed that FT individual motility (23.8% vs 25.8%), and sHOST (10.5 vs 11.5%) between 0.5 and 0.25 mL straws were not differ significantly, whereas the percentages of viable (24.0 vs 31.4%) and normal acrosome (15.8 vs 28.5%) spermatozoa were favorable for 0.5 mL straws ($P<0.05$). These might be due to the freezing and/or thawing protocols used in this study were inappropriate to 0.25 mL straws. The addition of Equex-STM, detergent, to a freezing extender improved ($P<0.001$) boar sperm membrane integrity (7.8 vs 31.4%), motility (6.3 vs 25.8%), and normal acrosomes (3.8 vs 28.5%) after cryopreservation (3).

**Acknowledgement:** This study is granted by the Research and Development Center for Livestock Production Technology, Chulalongkorn University.

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# BIRTH OF PIGLETS AFTER INTRA-UTERINE ARTIFICIAL INSEMINATION WITH FROZEN-THAWED BOAR SEMEN IN THAILAND

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## Introduction

Frozen-thawed (FT) semen in pig has been developed many years ago (3). However, the uses of FT semen under field condition were limited due to low fertility rate and unpractical. Recently, intra-uterine artificial insemination (AI) technique in pig was developed in order to reduce number of spermatozoa per insemination dose and increases the fertility rate of FT semen (2). The aim of the present study was to assess fertility result of FT boar semen produced in Thailand after intra-uterine AI in sows.

## Materials and methods

Three purebred-boar (1 Landrace, 1 Duroc and 1 Yorkshire) were used as semen donors. Collected semen was processed and frozen using a lactose-egg yolk-based extender with 3% glycerol and $1x10^9$ spz/mL in 0.5 mL straws according to the method described by Westendorf (3) with some modification (1). For insemination process: 7 post weaned sows, parity 3-4, were detected for standing estrus twice a day by back pressure test with boar contact. These sows were inseminated at 24 h after the onset of estrus by deep intra-uterine artificial insemination (DIUI) with $1x10^9$ spz/dose (2 sows) and intra-uterine artificial insemination (IUI) with $2x10^9$spz/dose (5 sows). The sows were re-inseminated at 12 h later. During insemination period, ovulation time were monitored by transrectal ultrasonography. Pregnancy detection was performed by trancutaneous ultrasonography at 28–30 d post service. At farrowing, litter sizes, sex and body weight of the piglets were recorded.

## Results and Discussion

At 30 d post service, 5 out of 7 sows inseminated (2 DIUI and 3 IUI sows) became pregnant (71.4%). All pregnant sows farrowed normally. In the present study, an inappropriate time of insemination in relation to ovulation time and early embryonic death were suspected as causes of pregnancy failure in 2 sows. The average total number of piglets born per litter was 9.4 (ranged 8-12), comparable to result of the previous study (2). A total ratio of male:female piglets was 1.2:1, and a mean piglets' weight was 1.1 kg. FT boar semen seemed to have no effect on sex and body weight of piglets. Nevertheless, it needs to be further investigated.

In conclusion, FT boar semen produced in Thailand can generate live offspring, in satisfying level, after being used to deeply inseminate in sows.

**Acknowledgement:** This study is granted by the Research and Development Center for Livestock Production Technology, Chulalongkorn University.

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