CHAPTER III

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MO, USA, otherwise specified.

Experimental design

Experiment 1

This experiment was performed to compare the effect of freezing techniques (conventional versus controlled-rate cryopreservation) on post-thawed sperm quality. Ejaculates were collected from three fertility-proven stallions and transported to the laboratory. The semen was equally divided and cryopreserved using either conventional freezing technique in a styrofoam box or programmable controlled-rate freezer. To determine semen quality, sperm motility, viability and longevity of the sperm was examined at 10 min, 2 and 4 hrs after thawing.

Experiment 2

This experiment was designated to test the type of sugars in freezing extenders that would be more favorable to protect sperm against freezing induced cryo-damage of stallion spermatozoa. Semen was obtained from six fertile stallions. In this experiment, programmable controlled-rate cryopreservation was used as *experiment 1* because programmable controlled-rate cryopreservation of stallion sperm yielded better post-thawing sperm quality when compared with conventional technique. The semen from each ejaculate was divided into three parts, and then separately cryopreserved with freezing medium containing with different types of sugar (glucose or fructose or sorbitol) as described in Table 3. After freezing and thawing, the sperm quality in terms of sperm motility, viability, acrosome integrity, functional plasma membrane and sperm longevity test at 10 min, 2 and 4 hrs post-thawing were examined.

Table 3. Compositions of freezing extenders used in this experiment

Ingredients	Ext F	Ext G	Ext S
Washing and centrifugation (solution I)			
Fructose (mM)	333.0	-	-
Glucose monohydrate (mM)	-	333.0	-
Sorbitol (mM)	-	-	333.0
Tri-sodium citrate (mM)	12.6	12.6	12.6
Di-sodium EDTA (mM)	9.9	9.9	9.9
Sodium hydrogencarbonate (g)	14.3	14.3	14.3
Antibiotics	100 IU/ml penicillin and 100 µg/ml streptomycin		
Distilled deionized water (ml)	100.0	100.0	100.0
pH and osmolality were adjusted to 6.7 ± 0.1 and 400 ± 10 mOsm./kg respectively.			
Freezing extender (solution II)			
Lactose 11% (w/v) (ml)	50.0	50.0	50.0
Solution I with fructose (ml)	26.0	-	-
Solution I with glucose (ml)	-	26.0	-
Solution I with sorbitol (ml)	-	-	26.0
Egg yolk (ml)	20.0	20.0	20.0
Equex STM (ml)	0.5	0.5	0.5
Glycerol (ml)	4.0	4.0	4.0
pH and osmolality were adjusted to 7.0 ± 0.1 and 300 ± 10 mOsm./kg respectively.			

Animals

Three (*experiment 1*) and six (*experiment 2*) fertility-proven stallions (Appaloosa, Arabian, Quarter horse, Standard bred, Thoroughbred and Warmblood), aged between 5 to 10 years old were used. All stallions were maintained at grass and fed supplementary with concentrates. The stallions were access to water *at libitum*. The semen was routinely collected (approximately 1-2 times a week) and evaluated for semen quality. All semen are used for cryopreservation must be a criteria of $\geq$ 70% motile, viable and morphologically normal sperm.

Semen collection

Three to four ejaculates were collected from each stallion, using artificial vagina (Hanover model, Germany) as described by Theerawat Tharasanit et al. (2007). In brief, artificial vagina (AV) was filled with 1.5 liters of 45-50 °C warm water. The internal pressure of the AV was adjusted to be suitable for each individual stallion. After semen collection, semen was filtered through sterile gauze and then examined for volume, pH and concentration. Raw semen was diluted 1: 3 with non-fat dry milk glucose based semen extender (pH 7.35, 350 mOsm) and transported to the laboratory at 4-6 °C in a Styrofoam box.

Freezing extenders

Freezing extenders used in this study were classified into 3 groups based on different sugars as described in Table 3. The pH and osmolality of the freezing extenders were measured by a pH meter (EUTECH Instruments pH 510, Singapore) and micro-osmometer (The FISKE® Micro-Osmometer model 210, USA.), respectively.

Semen cryopreservation and thawing

After 4 hrs of transportation, the semen was divided into 3 groups and centrifuged at 400 g for 10 min at 4°C (Jasko et al., 1992; Bedford et al., 1995). The sperm pellet was washed once with each type of washing solution (either glucose or fructose or sorbitol EDTA semen extender). After centrifugation, the three different sugars in freezing extenders containing 4% (v/v) glycerol was gently added to make a final concentration of 200 x 10^6sperm/ml. The sperm suspension was equilibrated at 4 °C for 60 min and loaded into 0.5 ml freezing straw. In *experiment 1*, conventional freezing technique was performed by placing the straw containing semen on a metal rack, 4 cm above liquid nitrogen. After 10 min, the straws were plunged into liquid nitrogen and kept for approximately 1-2 weeks before semen analysis. Programmable controlled-rate freezing technique was performed by placing the straw vertically into a cryochamber of freezing apparatus (Cryologic system, Australia). The freezing curve was controlled by Cryogenesis freezing program (version 5, Australia). The initial temperature was set at 4 °C, at which the temperature was reduced to -8°C at a rate of -6 °C/min. After 5 min at this temperature, the temperature was then gradually reduced from -8°C down to -50 °C at a rate of -4 °C/min.

Thawing of frozen semen was carried out at 37 °C in water bath for 30 sec (Borg et al., 1997). After incubation of the frozen-thawed semen at 37 °C for 10 min and 1:1 dilution of frozen-thawed semen with non-fat dry milk extender (NFDM), the semen was centrifuged at 400 g for 10 min at 25°C (Jasko et al., 1992, Bedford et al., 1995). The supernatant was discarded and the pellet was resuspened with fresh NFDM, in order to remove cryoprotectants that may render cytotoxic effect of glycerol on sperm viability during incubation.

Assessment of sperm quality

Sperm morphology

Head morphology examination was assessed using William's staining method and examined under a light microscope at 1000x magnification. For sperm tail morphology, 5-10 µl sperm suspension diluted with formal saline solution at 1:10 dilution ratios was placed on a grass microscopic slide and then examined under a light microscope (Olympus, Japan) at 400x magnification. A total of 200 spermatozoa were examined for each sperm quality test. Sperm head and tail morphology including loose head, acrosome defect, mid-piece defect, proximal and distal cytoplasmic droplets, bent tail and coiled tail were classified as abnormal sperm.

Concentration

Sperm concentration was assessed by a hemocytometer following dilution (1:100 v/v with formal saline solution). Ten microliters of diluted semen was loaded into the chamber and the number of sperm was evaluated under a phase contrast microscope (Olympus, Japan) at 400x magnification. Sperm concentration was expressed as the number of sperm $x10^6$ per ml.

Progressive motility

Sperm motility was estimated at before freezing (post-equilibration of semen at 4°C for 60 min.) and 10 min., 2 and 4 hrs after thawing (semen maintained at room temperature about 20-25°C). Progressive motility of sperm was estimated by placing a 10 µl drop of the sperm suspension onto a pre-warmed glass microscopic slide at 37°C and examined under a phase contrast microscope (Olympus, Japan) at 200x and 400x magnification. Motility examination was subjectively assessed by an experience person. Motility was expressed as the percentage of motile sperm cells.

Viability and plasma membrane integrity

Sperm viability was evaluated using multiple fluorescent probes labeling. Integrity of sperm plasma membrane was assessed by a non-membrane permeable DNA staining (Ethidium homodimer-1, EthD-1, Molecular Probes™, Oregon, USA), while esterase enzyme activity of the sperm was examined by staining the sperm with calcein AM (Molecular Probes™, Oregon, USA) (Borg et al., 1997). A total of 200 spermatozoa were evaluated and classified into 2 categories as either viable (intact plasma membrane: positive to calcein AM and negative to EthD-1) or dead sperm (damaged plasma membrane, EthD-1 positive).

Acrosome integrity

The integrity of sperm acrosome was evaluated by fluorescein isothiocyanate–labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) combined with Ethidium homodimer-1 (EthD-1) staining as described by Cheng et al. (1996). Briefly, semen sample (10 µl) was mixed with 10 µl of 4µM EthD-1 in PBS and incubated for 15 min at 37 °C. Then, 5 µl of solution was smeared on a glass slide and air dry. The sample was fixed with 96 % ethanol for 30 second and air dry. To stain acrosomal membrane of the sperm, 20 µl of 100 µM FITC-PNA (dilute FITC-PNA with PBS 1:10) was spreaded over the slide and incubated in a moist chamber at 4°C for 30 min. The sample was subsequently rinsed with cold PBS and air dry. Only viable sperm that negative to EthD-1 staining was examined under a fluorescent microscope (BX51, Olympus, Japan) at 1000x magnification. The sperm was classified as intact acrosome, partially and damaged acrosome.

Functional integrity of plasma membrane

Functional integrity of sperm plasma membrane was assessed using a short hypoosmotic swelling test (sHOST) as essentially described by Neild et al. (1999). In short, semen sample was incubated at 37 °C for 30 min, with a hypo-osmotic solution (100 mOsm/kg) consisting of 0.49 % (w/v) Na_3-citrate and 0.9 % (w/v) fructose (Merck, Germany) in distilled water. Following this incubation time, the semen was fixed in

hypoosmotic solution supplemented with 5% formaldehyde (Merck, Germany). The types of typical tail abnormalities indicative of swelling (sHOST positive) were evaluated under light microscopy at 400x magnification.

Longevity test

After freezing and thawing, sperm diluted with NFDM extender as described previously was incubated at room temperature (25°C) (Kenney et al., 1983; Varner et al., 2002) and assessed for sperm quality at 10 min, 2 and 4 h post-thawing, in terms of motility, viability, acrosome integrity.

Fertility assessment of cryopreserved-thawed stallion semen

This part of the study was performed to test fertility of cryopreserved stallion semen *in vivo* by means of pregnancy rate after artificial insemination. Fertility test of frozen-thawed semen obtained from the best semen extender (sorbitol) was determined in four mares. Estrous induction was performed by intramuscular injection of 375 µg of cloprostenol sodium (Estrumate™, Intervet, The Netherlands) in 4 diestrus mares. Estrus mares with $\geq$ 3.5-4.0 cm. follicle were received human chorionic gonadotropin (hCG, Intervet-Schering-Plough, NJ, USA) to induce ovulation. Mare's ovaries were monitored every 4-6 hrs after hCG injection using transrectal ultrasonography (HS-2000, Honda, Aichi, Japan). Artificial insemination with frozen semen was performed after ovulation using flexible deep intrauterine catheter (Minitube, Germany). Only frozen-thawed sperm that had more than 40% progressive motility with approximately $500x10^6$ motile sperm were used for artificial insemination. The pregnancy was performed at approximately 14-35 days after ovulation.

Statistical analysis

Data was analyzed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., Cary, NC, USA). Descriptive statistics were used to describe the semen quality before and after cryopreservation. The semen quality before freezing and after thawing was compared between conventional and controlled-rate freezing techniques using independent t-test. Pearson's correlation was used to evaluate the correlation among all measured sperm parameters that are sperm motility, viability, acrosome integrity and functional integrity of sperm plasma membrane. Comparisons between sperm quality parameters of each stallion were analyzed using analysis of variance (ANOVA) with the General Linear Model (GLM) to test the fixed effects of stallion, freezing extenders (glucose, fructose and sorbitol) and sperm quality at each time point considering all interaction terms. A *Bonferroni test* was used to determine differences between individual semen quality variables. All *P*-values <0.05 were considered statistical significance.