African Journal of Biotechnology Vol. 11(15), pp. 3697-3703, 21 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4022
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Improvement of the frozen boar semen quality by docosahexaenoic acid (DHA) and *L*-cysteine supplementation

Panida Chanapiwat[1], Kampon Kaeoket[2]*, and Padet Tummaruk[1]

[1]Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand 10330.
[2]Semen Laboratory, Department of Clinical Science and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand 73170.

Accepted 1 February, 2012

The aim of the present study was to investigate the effect of docosahexaenoic acid and *L*-cysteine supplementation on qualities of cryopreserved boar semen. A total of 30 ejaculates from 10 Yorkshire boars were included in the study. The semen was cryopreserved in lactose egg yolk base extender containing different concentrations of docosahexaenoic acid (fish oil) or/and *L*-cysteine : 0 mg and 0 mM (Control), 290 mg and 0 mM (Treatment I), 290 mg and 5 mM (Treatment II) and 290 mg and 10 mM (Treatment III). Post thawing semen qualities (sperm motility, motility patterns, sperm viability, acrosomal integrity, functional plasma membrane integrity and DNA damage) were assessed. The results show that sperm motility and sperm viability were significantly improved by supplementing of docosahexaenoic acid and/or *L*-cysteine, or their combination if compared to the control group ($P < 0.05$). The improvement of sperm parameters after supplementation of docosahexaenoic acid or/and *L*-cysteine, or their combination was more pronounced in the poor freezability sperm rather than at the good one. In poor freezability sperm, the supplementation of docosahexaenoic acid and/or 10 mmol/l *L*-cysteine resulted with higher sperm motility (34.7 vs. 22.3%, $P < 0.001$), sperm viability (45.0 vs. 28.1%, $P < 0.05$) and acrosomal integrity (46.9 vs. 36.2%, $P < 0.05$) if compared to the control. In conclusion, the supplementation of docosahexaenoic acid, alone or in combination with *L*-cysteine significantly enhanced the sperm motility, sperm viability and acrosomal integrity of boar sperm after cryopreservation.

Key words: Boar, docosahexaenoic acid, frozen semen, *L*-cysteine.

INTRODUCTION

During cryopreservation, cryoinjuries of the sperm are caused by physical and chemical factors including rapid change in temperature (also known as thermal stress), intracellular ice formation, oxidative stress and osmotic stress. These events lead to the damage of the sperm plasma membrane and subsequently reduced post thawing individual motility and fertilizing ability (Medeiros et al., 2002; Chanapiwat et al., 2009). Characteristics of the sperm cryoinjury include destabilization of the sperm plasma membrane lipid bilayer, changes in permeability of plasma membrane and reduction of the sperm viability (Watson, 2000; Rodriguez-Martinez, 2009). It is well documented that the boar sperm is highly susceptible to the temperature below 15°C, mainly due to its lipid composition and the structure of plasma membrane if compared to other domestic species (Parks and Lynch, 1992; Medeiros et al., 2002). The boar sperm plasma membrane consists of high levels of poly-unsaturated fatty acids (PUFAs), especially docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) and low level of cholesterol phospholipids ratio. It has been shown that PUFA level content plays an important role in the sperm membrane fluidity and causes sperm susceptible to lipid peroxidation (LPO) (Darin-Bennette and White, 1977;

*Corresponding author. E-mail: vskkk@mahidol.ac.th. Tel: +66 2 4415242. Fax: +66 2 4410937.

Aitken et al., 1994). During freezing and thawing (FT) processes, sperms are attacked by reactive oxygen species (ROS), owing to LPO and leading to significant decrease in the PUFAs content of their plasma membrane (de Lamirande and Gagnon, 1992; Chatterjee et al., 2001). Reactive oxygen species were mainly produced by the defective or dead spermatozoa and resulted in the reduction of sperm motility, sperm viability and eventually decreased fertilizing ability (Agarwal et al., 2005; Buranaamnuay et al., 2010). The supplement of antioxidant compounds and some fatty acid to the semen extender have been reported to minimize ROS formation and enhance the plasma membrane function in many species (Penã et al., 2003; Gadea et al., 2004; Roca et al., 2004, 2005; Maldjian et al., 2005; Malo et al., 2010). *L*-Cysteine, a precursor of intracellular glutathione plays an important role in protecting of the sperm from oxidative stress and acts as capacitation inhibitor (Johnson et al., 2000). Earlier studies have demonstrated that *L*-cysteine supplement in the semen extender improves motility of the FT bull semen (Bilodeau et al., 2001), prolongs sperm survival time and reduces chromatin damage in the FT boar sperm (Szczesniak-Fabianczyk et al., 2003). In addition, the n-3-enriched hen egg yolk in the semen extender increases proportion of DHA content in the boar sperm (Maldjian et al., 2005). Our previous study showed that addition of *L*-cysteine directly into the lactose egg yolk (DHA-enriched) base extender significantly improves sperm motility and intact acrosome of the FT boar sperm (Chanapiwat et al., 2009). Kaeoket et al. (2010a) found that the supplement of DHA (fish oil) improves the sperm motility, viability and acrosomal integrity of the FT boar sperm. The objective of the present study was to investigate the effect of DHA supplementation, alone or in combination with *L*-cysteine on the qualities of cryopreserved boar semen.

MATERIALS AND METHODS

The proposal of the present study was approved by the Institutional Animal Care and Use Committee (IACUC) (Approval No. 1031018), Chulalongkorn University.

Animals and semen collection

Ten Yorkshire boars aged between 1 to 3 years, having proven fertility and being used for routine AI in commercial herd were used in the present study. The boars were housed in individual pens, in closed housing facility with evaporative cooling system. Water was provided *ad libitum* via water nipple. Animals were fed twice a day 3 kg of corn-soybean-fishmeal base containing 15 to 16% of protein. A total of 30 ejaculates (3 ejaculates per boar) were collected, using gloved-hand method. Semen was collected once a week. Only sperm rich fractions were collected and evaluated within 30 min following collection. Semen volume, pH, subjective motility, sperm concentration, percentage of alive and dead sperm, and sperm morphology were determined. Only ejaculates with motility of ≥70 and ≥ 80 % and morphologically normal were used for cryopreservation.

Semen freezing and thawing procedures

Ten minutes following collection, semen was extended (1:1 v/v) with commercial extender (Modena™, Swine Genetics International, Ltd., Iowa, USA). The extended semen was cooled down to 15°C for 120 min and centrifuged at 800 × g for 10 min. Following centrifugation, the sperm pellet was re-suspended with freezing extender I (80% v/v lactose solution, 20% v/v egg yolk) to concentration of 1.5×10^9 sperm/ml. Afterwards, diluted semen was divided into the 4 groups according to the composition of freezing extender I (see below). After cooling at 5°C for 90 min, the semen was slowly mixed with freezing extender II (89.5% of freezing extender I with 9% glycerol and 1.5% Equex-STM Paste; Nova Chemical Sales Inc., Scituate, MA, USA) to final concentration of 1.0×10^9 sperm/ml , and contained 3% glycerol (Gadea et al., 2004; Chanapiwat et al., 2009). The cooled semen was packed into 0.5 ml straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before being placed in the contact with nitrogen vapour, about 3 cm above the liquid nitrogen level for 20 min in the styrofoam box (Buranaamnuay et al., 2010), and plunged into liquid nitrogen (-196°C) for storage. Thawing was carried out at 50°C for 12 s (Selles et al., 2003). The semen was diluted (1:4) with a Modena™ extender. Post thawing sperm qualities were evaluated after incubation at 37°C waterbath for 15 min.

Freezing extender and treatments

In the presented study, the semen was divided into 4 groups according to the composition of freezing extender I. In the control, the freezing extender I contained 80 ml of 11% lactose solution and 20 ml egg yolk without supplementation. Treatment I was supplemented with 290 mg of fish oil (Fish oil 1000®; Blackmores Ltd., New Southwell, Australia). The fish oil contained DHA 120 mg/g, while the normal egg yolk contained 3.15 mg DHA per gram of egg yolk. The DHA content in the normal egg yolk was analyzed at the Institute of Nutrition, Mahidol University (AOAC, 2007). Treatment II was supplemented with a combination of fish oil 290 mg and *L*-cysteine 5 mM (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland) while treatment III was supplemented with 290 mg of fish oil and 10 mM of *L*-cysteine

Sperm concentration and motility

Sperm concentration was assessed by direct cell count using Bürker haemocytometer (Boeco, Humburg, Germany) (Bearden and Fuquay, 1997). The sperm motility of both fresh and FT sperm was evaluated at 37°C under the phase contrast microscope at 200x and 400x magnification.

Computer-assisted sperm analysis (CASA)

The motility patterns of diluted FT semen were assessed using Computer-assisted sperm analysis (CASA) system (Hamilton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, USA). The FT semen samples were re-extended in the pre-warmed Modena™ extender at 37°C to the final concentration of 50×10^6 sperm/ml. A 5 µl of semen aliquot was pipetted into the chamber and left alone for 1 min prior to analyses. The CASA sperm assessment was carried out immediately (T0) and after 30 min (T30), and 60 min (T60) of incubation at 37°C in Modena™ extender. The sperm heads were marked using different colors to enable observer and analyzer to differentiate between the different motility patterns. Each semen sample was measured twice, 3 fields

were evaluated and at least 1,000 cells were counted at each analysis. The motility patterns included (1) Curvilinear velocity (VCL, μm/s), the average velocity measured in progression line along the whole track of cell path; (2) Average pathway velocity (VAP, μm/s) as the average velocity of the smoothed cell path; (3) Straight line velocity (VSL) as the average velocity measured in the straight line from the beginning to the end of the track (μm/s); (4) The amplitude of the lateral head displacement (ALH), as the mean width of the head oscillation as the sperm cells swim (μm); (5) The beat cross-frequency (BCF, Hz) as frequency of the sperm head crossing the average path in either direction; (6) The straightness (STR, %) average value of the ratio VSL/VAP; (7) The Linearity (LIN, %) = average value of the VCL/VAP ratio (Iguer-Ouada and Vestegen, 2001; Tretipskul et al., 2010)

Sperm viability

The proportion of live sperm was evaluated by SYBR-14/ Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherland). This technique was modified by Axnér et al. (2004) and Garner and Johnson (1995). Briefly, 10 µl of semen sample was diluted using 140 µl of thawing medium. Following this, 50 µl of diluted semen was mixed with 2.7 µl of SYBR-14 and 10 µl of EthD-1. After incubation at 37°C for 20 min, 200 spermatozoa were assessed (x1000) under fluorescent microscope. The nuclei of spermatozoa with intact plasma membrane were stained green with SYBR-14, while those with damaged membranes were stained red with EthD-1. Sperm were classified into two types; alive sperm stained green and dead sperm stained red.

Acrosomal integrity

The post thawing intact acrosome was assessed using fluorescein isothiocyanate–labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Ten µl of semen sample was diluted using 140 µl of thawing medium. Following that 10 µl of diluted semen was mixed with 10 µl of EthD-1and incubated at 37°C for 15 min. Five microliter (5 µl) of the mixture was smeared on the glass slide and fixed with 95% ethanol over 30 s. Fifty microliter (50 µl) of Fit C-PNA solution (100 µg/ml in PBS) was spread over each slide. Slides were incubated in a moist chamber at 4°C for 30 min. Following incubation, slides were rinsed with cold PBS and air dried. The minimum of 200 sperm were assessed under fluorescent microscope at 1,000x magnification and classified as intact acrosome, reacted acrosome and loose acrosome (Axnér et al., 2004; Cheng et al., 1996)

The functional integrity of the sperm plasma membrane

The proportion of the functional intact plasma membrane was assessed using a short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Five hundred microliter (500 µl) of sperm suspension were mixed using 500 µl hypo-osmotic solution (75 mOsm/kg) consisting of 0.368% (w/v) Na-citrate and 0.675% (w/v) fructose (Merck, Darmstadt, Germany) in distilled water. The mixture of semen and hypo-osmotic solution was incubated at 37°C over 5 min. Following this, 200 µl of the mixture was fixed in 1ml of a hypo-osmotic solution plus 5% formaldehyde (Merck, Darmstadt, Germany), for later evaluation. At least, 200 sperm were assessed under a phase contrast microscope at 400x magnification the sperm with coiled tail following incubation was defined as the functional intact plasma membrane (sHost Positive).

DNA damage

The DNA damage was evaluated using Acridine orange (AO) staining, which had been applied previously in pig (Chanapiwat et al., 2010). Five µl of sperm suspension was smeared on each slide. Two smeared slide were prepared air-dried and fixed overnight in freshly prepared Carnoy's solution (methanol and glacial acetic acid; 3:1 v/v). The slide was removed from the fixative solution, air-dried, and stained with 1% (100 mg/ml) AO (SIGMA-ALDRICH, Inc., Steinheim, Germany) in distilled water over 10 min. The AO staining solution was daily prepared by adding 10 ml of 1% AO in distilled water to 40 ml of 0.1 M citric acid (Merck, Darmstadt, Germany) and 2.5 ml of 0.3 M Na2HPO4.7H2O (Merck, Darmstadt, Germany) pH 2.5. After staining, the slide was washed using distilled water and air-dried. In total 1,000 sperm were evaluated under the fluo-rescence microscope. The heads of the sperm cells with normal DNA (double-stranded) had green fluorescence, while those with damaged or single stranded DNA showed orange or red fluorescence. The results were expressed as proportion of the damage/single stranded DNA per 1,000 counted sperm.

Statistical analysis

The statistical analysis was performed using Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). Normality of the data was evaluated using UNIVARIATE procedure option NORMAL PLOT. The sperm parameters not normally distributed (sperm motility, acrosome integrity, functional plasma membrane integrity and DNA damage) were transformed using arcsine transformation. Sperm motility, motility pattern measured by CASA (7 parameters), sperm viability, acrosomal integrity and functional integrity of the sperm plasmal membrane were analyzed using General Linear Mixed Model (MIXED) procedure of the SAS. Group of extenders (control, treatment I, II and III) was included in the statistical model as a fixed effect and boar's ID (10 boars) and ejaculates (30 ejaculates) nested within boar were included in the model as random effects. Sperm ejaculates were classified as "good" and "poor" freezability according to post thawing sperm motility. If post thawing sperm motility was higher than 35%, then the sperm ejaculate was classified as "good". If post thawing sperm motility was less than 35%, the sperm ejaculate was classified as "poor. In addition, statistical analysis was performed separately according to the freezability of the sperm. Statistically significant difference was defined as $P < 0.05$.

RESULTS AND DISCUSSION

The post thawing sperm motility, viability, acrosome integrity, functional plasma membrane integrity and DNA damage are presented in Table 1. The sperm motility in Treatment II and III was higher if compared to the control group ($P < 0.05$). The sperm viability in the control group was for 7.9, 6.8 and 10.8% lower if compared to the Treatment I, II and III, respectively ($P < 0.05$). However, no significant difference between the acrosomal integrity, functional plasma membrane integrity and DNA damage was observed among the treatment groups ($P > 0.05$). The motility patterns of the FT boar sperm in all groups are presented in Table 2. Average pathway velocity(VAP) of the FT boar sperm in Treatment II was higher if compared to the control group ($P < 0.05$).

Table 1. Means ± standard error of mean (SEM) of sperm motility (%), sperm viability (%), acrosomal integrity (%), sHost (%) and DNA damage (%) of post-thawing boar sperm between 4 groups (n=30 ejaculates per group).

Sperm parameter (%)	Group			
	Control (n=30)	Treatment I (n=30)	Treatment II (n=30)	Treatment III (n=30)
Sperm motility	26.7 ± 2.2[a]	32.7 ± 2.7[ab]	38.3 ± 2.2[b]	39.0 ± 1.9[b]
Sperm viability	32.3 ± 1.8[a]	40.2 ± 2.4[b]	39.1 ± 2.0[b]	43.1 ± 1.9[b]
Acrosomal integrity	41.2 ± 1.9[a]	44.6 ± 2.7[a]	44.9 ± 2.1[a]	47.6 ± 1.9[a]
sHost (%)[1]	16.7 ± 1.3[a]	16.4 ± 1.4[a]	17.9 ± 1.1[a]	18.4 ± 1.2[a]
DNA damage	2.2 ± 0.3[a]	2.2 ± 0.3[a]	3.4 ± 0.7[a]	3.1 ± 0.3[a]

[1]sHost = functional integrity of sperm plasma membrane.
[a,b]Values in each row marked with different letter in superscript differ significantly ($P < 0.05$).

Table 2. Least square means (LSM) ± standard error of mean (SEM) of post thawing sperm motility patterns evaluated by CASA after thawing immediately (n=30 ejaculates per group)

Motility pattern	Group			
	Control (n=30)	Treatment I (n=30)	Treatment II (n=30)	Treatment III (n=30)
LIN (%)	42.4 ± 1.5[a]	42.8± 1.5[a]	44.4 ± 1.5[a]	44.2 ± 1.5[a]
VSL (µm/sec)	50.3 ± 1.8[a]	51.3 ± 1.8[a]	51.9 ± 1.8[a]	52.7 ± 1.8[a]
VAP (µm/sec)	68.5 ± 6.3[a]	71.1 ± 6.3[a]	91.0 ± 6.3[b]	71.8 ± 6.3[a]
VCL (µm/sec)	116.9 ± 10.1[a]	122.4 ± 10.1[a]	121.9 ± 10.1[a]	123.3 ± 10.1[a]
ALH (µm)	7.1 ±0.2[a]	7.2 ±0.2[a]	7.0 ±0.2[a]	7.2 ±0.2[a]
BCF (Hz)	27.6±0.6[a]	27.6±0.6[a]	26.6±0.6[a]	27.2±0.6[a]
STR (%)	69.0 ±1.2[a]	69.5 ±1.2[a]	69.8 ±1.2[a]	70.4 ±1.2[a]

LIN, Linearity; VSL, straight line velocity; VAP, average pathway velocity; VCL, curvilinear velocity; ALH, amplitude of the lateral head displacement; BCF, beat cross-frequency; STR, straightness.
[a,b]Values in each row marked with different letter in superscript differ significantly ($P < 0.05$).

The post thawing quality of boar sperm between good and poor freezability are presented in the Table 3. In the poor freezability sperm, supplementation of DHA and *L*-cysteine (treatment III) resulted in higher sperm motility, sperm viability and acrosomal integrity if compared to the control group ($P < 0.05$). However, the proportion of functional plasma membrane and DNA damage were not significantly different among the treatment groups (Table 3). In good freezability sperm, sperm motility in Treatment III increased for 12.3% if compared to the control group ($P < 0.05$).Yet, no significant difference within the sperm viability, acrosomal integrity, functional plasma membrane integrity and DNA damage was found among the treatment groups ($P > 0.05$).

The present study demonstrated that supplementation of DHA either alone or combined with *L*-cysteine significantly improved total motility and sperm viability of the FT boar sperm, and is in accordance with some earlier studies (Chanapiwat et al., 2009; Kaeoket et al., 2010b). It is evident that sperm plasma membrane contains high amounts of PUFAs, especially DHA (Johnson et al., 1969; Park and Lynch, 1992). PUFAs play a critical role in the regulation of the sperm maturation, spermatogenesis, capacitation, acrosome reaction and sperm-oocyte membrane fusion in many species (Parks and Hammerstedt, 1985; Martinez and Morros, 1996). It has been shown that freezing extender contains DHA-enriched hen egg yolk, resulting with higher motility and acrosomal integrity of the FT boar sperm (Chanapiwat et al., 2009). This also indicates that the ability of the sperm plasma membrane to uptake and utilize DHA in the freezing extender consequently increases the membrane fluidity resulting with increase of the FT sperm quality in the present study.

During cryopreservation processes, it has been demonstrated that the cooling, freezing and thawing processes increases ROS production and oxidative damage of the sperm plasma membrane, which in turn impaired structure and function of sperm (de Lamirande and Gagnon, 1992; Chanapiwat et al., 2010). In order to minimize the ROS, the antioxidant supplementation, for example, alpha-tocopherol (Penã et al., 2003; Kaeoket et al., 2008), glutathione peroxidase (Gadea et al., 2004; Roca et al., 2005), *L*-glutamine (de Mercado et al., 2009) and *L*-cysteine (Chanapiwat et al., 2010; Kaeoket et al., 2010b) have been suggested. In boar, it has been reported that supplementation of *L*-cysteine alone to the freezing extender at concentration of 5 mM or 10 mM

Table 3. Means ± standard error of mean (SEM) of sperm motility (%), sperm viability (%), acrosomal integrity (%), sHost (%) and DNA damage (%) of post thawing boar sperm (Good freezability and poor freezability) between 4 groups.

Sperm parameter (%)	Frozen-thawed Semen							
	Poor freezability				Good freezability			
	Control (n=15)	Treatment I (n=15)	Treatment II (n=15)	Treatment III (n=15)	Control (n=15)	Treatment I (n=15)	Treatment II (n=15)	Treatment III (n=15)
Sperm motility	22.3 ± 2.9[a]	26.7 ± 3.0[ab]	30.3 ± 2.9[ab]	34.7 ± 2.9[b]	31.2 ± 2.9[a]	38.6 ± 4.0[a]	46.3 ± 1.9[b]	43.3 ± 1.7[b]
Sperm viability	28.1 ± 2.1[a]	36.8 ± 3.3[ab]	36.5 ± 3.1[ab]	45.0 ± 3.4[b]	36.4 ± 2.5[a]	41.8 ± 3.6[a]	41.4 ± 4.0[a]	42.8 ± 1.9[a]
Acrosomal integrity	36.2 ± 11.9[a]	40.1±12.9[ab]	39.3 ± 2.0[ab]	46.9 ± 3.5[b]	46.3 ± 2.7[a]	49.3 ± 3.9[a]	51.6 ± 2.9[a]	48.3 ± 2.2[a]
sHost [1]	15.2 ± 8.9[a]	12.3 ± 7.4[a]	16.1 ± 1.5[a]	18.7 ± 1.6[a]	18.5 ± 2.4[a]	17.9 ± 1.7[a]	20.1 ± 1.5[a]	18.1 ± 1.8[a]
DNA damage	2.2 ± 0.4[a]	2.3 ± 0.3[a]	3.2 ± 0.4[a]	3.1 ± 0.5[a]	2.3 ± 0.3[a]	2.0 ± 0.6[a]	3.6 ± 1.3[a]	3.1 ± 0.4[a]

[1]sHost = functional integrity of sperm plasma membrane.
[a,b]Values in each row marked with different letter in superscript differ significantly ($P < 0.05$).

may improve the FT sperm qualities (Kaeoket et al., 2010b). In addition, *L*-cysteine has been constituent in the freezing extender for cryopreservation in other species including the dog (Michael et al., 2007), cat (Thuwanut et al., 2008), bull (Bilodeau et al., 2001) and ram (Uysal and Bucak et al., 2007). It is well documented that *L*-cysteine acts as the membrane stabilizer and capacitation inhibitor (Johnson et al., 2000), as well as precursor of intracellular glutathione (GSH), a cofactor in important enzymatic antioxidants, that is, glutathione peroxidase, glutathione reductase in which it protects lipids, proteins and nucleic acids from oxidative stress by detoxification of hydrogen peroxides (Bilodeau et al., 2001). Freezing and thawing process resulted with significant decrease in the glutathione level of the boar (Gadea et al., 2004) and bull semen (Bilodeau et al., 2001). Consequently, the supplement of *L*-cysteine in the freezing extender may increase the intracellular GSH levels and provide protective effect on the FT boar sperm as evident in the present results. In the present study, the supplementation of *L*-cysteine at concentration of 5 mM or 10 mM in DHA containing freezing extender increased FT sperm motility and sperm viability. To our knowledge, the combination effect of DHA and *L*- cysteine on the FT boar sperm qualities had not been elucidated. Regarding the sperm freezability, the underlying mechanism of differences between boars and ejaculates with good and poor freezability have been described by evaluation of the chromatin structure, the kinematics parameters, mitochondrial activity and fertilization rate of the FT sperm (Gil et al., 2005; Hernandez et al., 2006). It has also been demonstrated that the good freezability sperm showed significantly higher rates of *in vitro* and *in vivo* fertilization than the poor freezability sperm (Gil et al., 2005; Casas et al., 2010). Interestingly, in the present study, we found that DHA combined with 10 mM *L*-cysteine not only significantly increased the sperm motility in good freezability sperm, but also enhanced the total sperm motility, sperm viability and acrosomal integrity in the poor freezability sperm. Therefore, the concentration of DHA and *L*-cysteine used for studying the good and poor freezability of boar sperm is considered important. In addition, the reason for individual boar difference in freezability of sperm might be related with genetic background and the difference in the lipids or proteins composition in the sperm plasma membrane among boars (Holt et al., 2005; Thurston et al., 2002). It could be concluded that the supplementation of DHA, alone or in com-bination with L-cysteine, significantly enhanced the sperm motility and viability of boar sperm following cryopreservation.

ACKNOWLEDGEMENTS

The present study was financially supported by the Thailand Research Fund (IUG5080002) and partly by National Research Council of Thailand (NRCT). P. Chanapiwat is a grantee of Commission on Higher Education (CHE-50).

REFERENCES

Aitken RJ, West KM, Buckingham DW (1994). Leukocyte infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. J. Androl. 15: 343-352.

Agarwal A, Prabakaran SA, Said TM (2005). Prevention of oxidative stress injury to sperm. J. Androl. 26: 102-660.

Axnér E, Hermansson U, Linde-Forsberg C (2004). The effect of Equex STM paste and sperm morphology on frozen-

thawed survival of cat epididymal sperm. Anim. Reprod. Sci. 84: 179-191.
Bearden HJ, Fuquay JW (1997). Semen evaluation. In: Applied Animal Reproduction (Bearden HJ, Fuquay JW eds.), Prentice Hall, New Jersey, USA. pp. 159-170.
Bilodeau JF, Blanchette S, Gagnon C, Sirard MA (2001). Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology, 56: 275-286.
Buranaamnuay K, Tummaruk P, Singlor J, Rodriguez-Martinez H, Techakumphu M (2009). Effects of straw volume and Equex-STM on boar sperm quality after cryopreservation. Reprod. Domest. Anim. 44: 69-73.
Buranaamnuay K, Wongtawan T, Masuwatana S, Tummaruk P, Techakumphu M (2010). Intra-uterine and deep intra-uterine insemination using cryopreserved boar semen in spontaneously-ovulating sows. Thai. J. Vet. Med. 40: 215-219.
Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, Bonet S (2010). Fertility after post-cervical artificial insemination with cryopreserved sperm from boar ejaculates of good and poor freezability. Anim. Reprod. Sci. 118: 69-76.
Chanapiwat P, Kaeoket K, Tummaruk P (2009). Effects of DHA-enriched hen egg yolk and L-cysteine supplementation on quality of cryopreserved boar semen. Asian. J. Androl. 11: 600-608.
Chanapiwat P, Kaeoket K, Tummaruk P (2010). The sperm DNA damage after cryopreservation of boar semen in relation to post-thawed semen qualities, antioxidant Supplementation and boars Effects. Thai. J. Vet. Med. 40: 187-193.
Chatterjee S, De Lamirande E, Gagnon C (2001). Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol. Reprod. Dev. 60: 498-506.
Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B (1996). Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J. Androl. 17: 674-682.
Darin-Bennette A, White IG (1977). Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology 14: 466-470.
de Lamirande ED, Gagnon C (1992). Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes and II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J. Androl. 13: 368-386.
de Mercado E, Hernandez M, Sanz E, Rodriguez A, Gomez E, Vazquez JM, Martinez EA, Roca J (2009). Evaluation of L-glutamine for cryopreservation of boar spermatozoa. Anim. Reprod. Sci. 115: 149-157.
Gadea J, Selles E, Marco MA, Coy P, Matas C, Romar R, Ruiz S (2004). Decrease in gluthathione content in boar sperm after cryopreservation, effect of the addition of reduced glutathione to the freezing and thawing extender. Theriogenology, 62: 690-701.
Garner DL, Johnson LA (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 53:276-284.
Gil MA, Roca J, Cremades T, Hernandez M, Vazquez JM, Rodriguez-Martinez H, Martinez EA (2005). Does multivariate analysis of post-thaw sperm characteristics accurately estimate *in vitro* fertility of boar individual ejaculates? Theriogenology, 64: 305-316.
Hernandez M, Roca J, Ballester J, Vazquez JM, Martinez EA, Johannisson A, Saravia F, Rodriguez-Martinez H (2006). Differences in SCSA outcome among boars with different sperm freezability. Int. J. Androl. 29: 583-591.
Holt WV, Medrano A, Thurston LM, Watson PF (2005). The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology, 63: 370-382.
Iguer-Ouada M, Verstegen JP (2001). Evaluation of the Hamilton Thorne computer-based automated system for dog semen analysis. Theriogenology, 55: 733-749.
Johnson LA, Gerrits RJ, Young EP (1969). The fatty acid composition of porcine spermatozoa phospholipids. Biol. Reprod. 1: 330-334.
Johnson LA, Weitze KF, Fiser P, Maxwell WMC (2000). Storage of boar semen. Anim. Reprod. Sci. 62: 142-172.
Kaeoket K, Tantiparinyakul K, Kladkaew W, Chanapiwat P, Techakumphu M (2008). Effect of different antioxidants on quality of cryopreserved boar semen in different breeds. Thai. J. Agric. Sci. 41: 1-9.
Kaeoket K, Sang-urai P, Thamniyom A, Chanapiwat P, Techakumphu M (2010a). Effect of Docosahexaenoic acid (DHA) on quality of cryopreserved boar semen in different breeds. Reprod. Dom. Anim. 45: 458-463.
Kaeoket K, Chanapiwat P, Tummaruk P, Techakampu M (2010b). Supplemental effect of varying L-cysteine concentrations on the quality of cryopreserved boar semen. Asian J. Androl. 12: 760-765.
Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL (2002). Current status of sperm cryopreservation: why isn't it better? Theriogenology, 57: 327-344.
Malo C, Gil L, Gonzalez N, Martínez F, Cano R, de Blas I, Espinosa E (2010). Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation:Comparison between cysteine and rosemary (*Rosmarinus officinalis*). Cryobiology, 61: 142-147.
Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R (2005). Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology, 63: 411-421.
Martinez P, Morros A (1996). Membrane lipid dynamics during human sperm capacitation. Front. Biosci. 1: 103-117.
Michael A, Alexopoulos C, Pontiki E, Hadjipavlou-Litina D, Saratsis P, Boscos C (2007). Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. Theriogenology, 68: 204-212.
Parks JE, Hammerstedt RH (1985). Development changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. Biol. Reprod. 32: 653-668.
Parks JE, Lynch DV (1992). Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. Cryobiology, 29: 255-266.
Penã FJ, Johannisson A, Wallgren M, Rodriguez Martinez H (2003). Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci. 78: 85-98.
Perez-Llano B, Lorenzo JL, Yenes P, Trejo A, Garcia-Casado P (2001). A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology, 56: 387-398.
Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA (2004). Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J. Androl. 25: 397-405.
Roca J, Rodriguez JM, Gil MA, Carvajal G, Garcia EM, Cuello C, Vazquez JM, Martinez EA (2005). Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J. Androl. 26: 15-24.
Rodriguez-Martinez H (2009). Freezing of boar semen: state of the art. Proceedings 4th Asian Pig Vet. Society, Tsukuba, Japan. pp. 55-58.
Selles E, Gadea J, Romar R, Matas C, Ruiz S (2003). Analysis of in vitro fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. Reprod. Dom. Anim. 38: 66-72.
Szczesniak-Fabianczyk B, Bochenek M, Smorag Z, Silvestre MA (2003). Effects of antioxidants added to boar semen extender on the semen survival and sperm chromatin structure. Reprod. Biol. 3: 81-87.
Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt W (2002). Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol. Reprod. 66: 545-554.
Thuwanut P, Chatdarong K, Techakumphu M, Axnér E (2008). The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology, 70: 233-240.
Tretipskul C, Buranaamnuay K, Koonjaenak S, Tummaruk P, Techakumphu M (2010). The use of computer-assisted sperm analysis for discriminating a series of motility pattern of frozen-thawed boar semen. Thai. J. Vet. Med. 40: 25-30.

Uysal O, Bucak MN (2007). Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta. Vet. Brno. 76: 383-390.

Watson PF (2000). The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61: 481-492.