npg

Original Article

Effects of DHA-enriched hen egg yolk and *L*-cysteine supplementation on quality of cryopreserved boar semen

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

[1]*Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Faculty of Veterinary Science, Mahidol University, Nakorn-Pathom 73170, Thailand*

Abstract

The objective of the present study was to determine the effects of docosahexaenoic acid (DHA)-enriched hen egg yolks and *L*-cysteine supplementation on the qualities of the cryopreserved boar semen. A total of 15 ejaculates from 5 Pietrain boars were divided into 4 groups according to the compositions of the freezing extenders used, that is, normal hen egg yolk (group I), DHA-enriched hen egg yolk (group II), normal hen egg yolk with 5 mmol L^{-1} of cysteine supplementation (group III) and DHA-enriched hen egg yolk with 5 mmol L^{-1} of cysteine supplementation (group IV). The semen was cryopreserved using controlled rate freezer and was thawed at 50°C for 12 s. Progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane of the post-thawed semen were evaluated. The supplementation of *L*-cysteine in the freezing extender alone (group III) improved progressive motility ($P < 0.05$), and the supplementation of *L*-cysteine in combination with DHA-enriched hen egg yolk (group IV) improved both progressive motility ($P < 0.05$) and acrosome integrity ($P < 0.01$). The use of DHA-enriched hen egg yolk alone (group II) did not enhance any of the post-thawed semen qualities ($P > 0.05$). In conclusion, the supplementation of antioxidant *L*-cysteine alone or in combination with DHA-enriched hen egg yolk significantly improved the post-thawed semen qualities, especially progressive motility and acrosome integrity.

1 Introduction

Cryopreservation of boar semen has been an established practice in the pig industry for decades [1, 2] in order to preserve genetic resources, enhance genetic improvement, distribute genetic material across countries and minimize boar transportation [3–5]. In general, frozen–thawed (FT) boar semen has a short survival time in the female reproductive tract compared with extended fresh semen (4 h *vs*. 24 h) [6], and has a low post-thawed semen quality, particularly regarding individual motility and plasma membrane integrity [7]. For these reasons, the use of FT semen under field conditions resulted in a decrease of 20%–30% in the conception rate and a reduction of two to four liveborn piglets per litter size compared with the use of extended fresh semen [8, 9]. Earlier studies have shown that the cryopreserved boar semen yielded at most 49%–53% individual sperm motility after thawing, and

each insemination required a total number of 5×10^9 spermatozoa per dose [8, 10].

During recent years, several improved methods for the cryopreservation of boar semen have emerged, including methods to affect the freezing–thawing rate [11, 12], freezing packages [11, 13–15] and the composition of the semen extenders [16–19]. Earlier studies have shown dramatic improvements of FT semen quality by use of these methods. For instance, supplementation of Equex in the semen extender yielded a percentage of individual sperm motility that was higher than conventional methods [15, 20, 21]. In addition, the packaging of semen in Flatpacks resulted in a 47% motility rate, which was higher than that in Maxi or Medium straws (34% and 36%, respectively) [22]. Despite the improvement of cryopreservation protocols over time, individual boar variation in the sperm freezability is still observed. The reason for individual boar variation in sperm freezability has not been completely elucidated [23, 24].

It is well established that the plasma membrane of boar spermatozoa is highly sensitive to thermal changes [25, 26]. The susceptibility to temperature shock is mainly because of poor lipid composition and structure of the plasma membrane of the boar spermatozoa [27]. The plasma membrane of boar spermatozoa contains high levels of polyunsaturated fatty acids (PUFAs), that is, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and has a low level of cholesterol: phospholipids ratio [28, 29]. It has been shown that the cholesterol: phospholipids ratio and the phospholipid saturation play an important role on the temperature shock [30]. PUFAs decrease dramatically when the spermatozoa are attacked by reactive oxygen species (ROS) because of lipid peroxidation (LPO) [31, 32]. ROS resulted from defective/dead spermatozoa, decreased progressive motility, increased mid-piece abnormalities and inhibited sperm–oocyte fusion [33, 34].

The supplementation of antioxidants and fatty acids in the semen extender has been shown to reduce ROS and improve plasma membrane integrity in stallions [35, 36], bulls [37, 38], rams [39, 40] and pigs [16–18, 41, 42]. It has been shown that DHA in boar semen increased after n-3-enriched hen egg yolks were added in the semen extender [42]. Recently, a study has shown that supplements of DHA from fish oil in the semen extender increased progressive motility, viability, plasma membrane integrity and acrosome integrity

of FT boar semen [19]. To date many of the DHA-enriched hen eggs have been produced in the hen-egg industry (for example, Dr Henn, Quality meat Co. Ltd, Thailand). In general, the DHA-enriched egg contained 200–250 mg of DHA, which is about twice higher than that contained in a normal egg. The use of DHA-enriched hen egg yolk for cryopreservation of boar semen has never been considered. In addition, supplementation of *L*-cysteine, a precursor of intracellular gluthathione biosynthesis, in the semen extender have been shown to increase individual sperm motility of FT bull semen [38], and increase sperm viability and acrosome integrity of the boar spermatozoa during chilled storage [43, 44]. *L*-cysteine plays a role in the intracellular protective mechanism against oxidative stress, and as a membrane stabilizer and capacitation inhibitor [10]. Both DHA and *L*-cysteine play a major role as the plasma membrane stabilizers of spermatozoa in many domestic species. The influence of *L*-cysteine and combination of *L*-cysteine and DHA on FT boar semen has never been investigated. Therefore, the present study investigates the influence of DHA-enriched hen egg yolk and *L*-cysteine in semen extender on the qualities of cryopreserved boar semen.

2 Materials and methods

The proposal of the present study has been approved by the Faculty of Veterinary Science Animal Care and Use Committee (FVS-ACUC), Mahidol University.

2.1 Boar and semen

Five Pietrain boars aged between 1 and 3 years were used in the experiment. The boars were of proven fertility and held in a commercial herd in Ratchaburi Province, Thailand. The boars were housed in individual pens with a conventional opened-house system in a commercial swineherd near to the laboratory (approximately 30 km). The feed was provided twice daily and water was available ad libitum. Semen was collected once weekly using the gloved-hand method. A total of 15 ejaculates of semen were obtained (3 ejaculates each). The semen was transported from the herd to the laboratory at the Faculty of Veterinary Science, Mahidol University. The semen volume, pH, individual sperm motility, sperm concentration, percentage of live and dead sperms, and morphology were evaluated. Ejaculates with a volume of > 100 mL, a pH of 7.2–7.8, a sperm motility of ≥ 70%, a sperm concentration of > 150 spermatozoa

per mL and $\geq 80\%$ normal sperm morphology were used.

2.2 Semen freezing and thawing procedures

Shortly after collection, the semen was diluted (1:1 [v/v]) using extender I (Modena, Swine Genetics International Ltd, Iowa, USA). The diluted semen was transferred to 50 mL centrifuge tubes, equilibrated at 15ºC for 120 min and centrifuged at $800 \times g$ for 10 min. The supernatant was discarded and the sperm pellet was re-suspended (about 1–2:1) using extender II (80 mL of 11% lactose solution and 20 mL egg yolk) to a concentration of 1.5×10^9 spermatozoa per mL. The diluted semen was cooled to 5ºC for 90 min. Then, two parts of the semen were mixed with one part of extender III (89.5% of extender II with 9% glycerol and 1.5% Equex-STM). The final concentration of semen was approximately 1.0×10^9 spermatozoa per mL and contained 3% glycerol (modified after Westendorf *et al.* [45] and Selles *et al.* [46]). The processed semen was loaded into 0.5 mL straws. The straws were sealed with PVC powder before being placed in a controlled rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria). The freezing rate was 3ºC min^{-1} from +5ºC to −5ºC and thereafter 50ºC min^{-1} from −5ºC to −140ºC. Then the straws were plunged into liquid nitrogen (−196ºC) for storage. Thawing was achieved by immersing the straws in water at 50ºC for 12 s [46]. Immediately after thawing, the semen was diluted (1:4) using a Modena extender. Post-thawed sperm qualities were evaluated after incubation in a 37ºC water bath for 15 min.

2.3 Semen extender

After incubation in extender I, the semen was divided into four groups according to the composition of extender II. In group I, extender II contained 80 mL of 11% lactose solution and 20 mL egg yolk. In group II, extender II was prepared by using DHA-enriched hen egg yolk (Dr Henn, Quality meat Co. Ltd Thailand) instead of conventional egg yolk. Each egg contained 200–250 mg of DHA. The DHA level as well as the fatty acid composition in the egg yolk that was used for preparing semen extender, was analyzed at the Institute of Nutrition, Mahidol University (Association of Official Analytical Chemists, Official Method Analysis, AOAC, Arlington, USA). On an average, DHA (C22 : 6, n-3) in the DHA-enriched hen eggs yolk was about 1.6 times higher than normal hen egg yolk (450 *vs.* 280 mg per

100 g). In group III, extender II was supplemented with 5 mmol L^{-1} of *L*-cysteine (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland). In group IV, extender II contained both DHA-enriched hen egg yolk and 5 mmol L^{-1} of *L*-cysteine.

2.4 Sperm evaluation
2.4.1 Sperm concentration and progressive motility

Sperm concentration was assessed by direct cell count using a Bürker hemocytometer (Boeco, Humburg, Germany) [47]. The visual progressive motility of both fresh and FT sperm was evaluated at 38ºC under a phase contrast microscope at × 200 and × 400 magnifications. The motility was assessed by a same person throughout the experiment.

2.4.2 Sperm viability

The viability was evaluated by SYBR-14 / ethidium homodimer-1 (EthD-1) (Fertilight, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands). This technique was modified after Axnér *et al.* [48]. In all, 10 µL of diluted semen was mixed with 2.7 µL of the user solution of SYBR-14 and 10 µL of EthD-1. The user solution was SYBR-14 diluted (1:100) in dimethyl sulfoxide, fractionated and frozen in eppendorfs. After incubation at 37ºC for 20 min, 200 spermatozoa were assessed (× 1 000) under fluorescent microscope. The nuclei of the spermatozoa with an intact plasma membrane were stained green using SYBR-14, whereas those with damaged membranes were stained red using EthD-1. Spermatozoa were classified into three types: live spermatozoa stained green using SYBR-14, dead spermatozoa stained red using EthD-1 and moribund spermatozoa stained both green and red (Figure 1) [48, 49]. The results are expressed as the percentage of live spermatozoa with intact plasma membranes.

2.4.3 Acrosome integrity

Acrosome integrity was evaluated using fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) staining. A total of 10 µL of the diluted semen was mixed with 10 µL of EthD-1 and incubated at 37ºC for 15 min. A total of 5 µL of the mixture was smeared on a glass slide and fixed with 95% ethanol for 30 s. FITC-PNA (dilute FITC-PNA with phosphate-buffered saline [PBS]; 1:10 [v/v]) (50 µL) was spread over the slide and incubated in a moist chamber at 4ºC for 30 min. After incubation, it was rinsed with cold PBS and air dried. A total of 200 spermatozoa were

Supplement of DHA-enriched hen egg yolk and *L*-cysteine
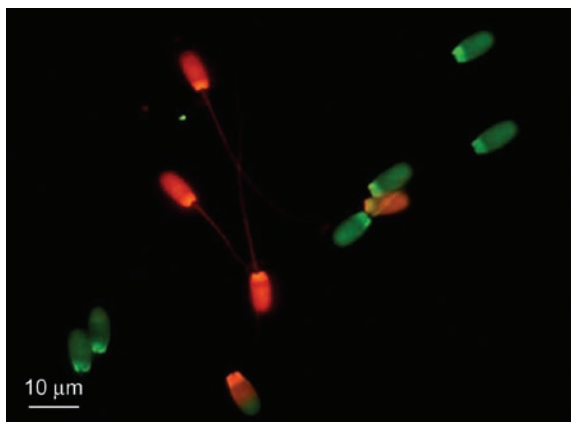Panida Chanapiwat *et al*.

npg

603

Figure 1. Boar spermatozoa stained with SYBR-14 / ethidium homodimer-1 (EthD-1). Live spermatozoa with intact plasma membrane are stained green using SYBR-14, dead spermatozoa are stained red using EthD-1 and moribund spermatozoa are stained both green and red.
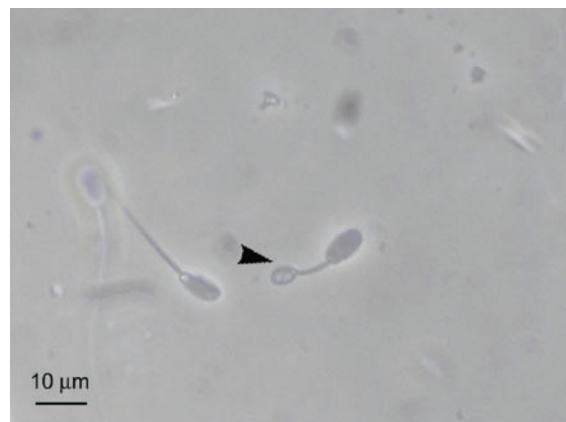

Figure 2. Boar spermatozoa in short hypoosmotic swelling test (sHOST test). Coiled tail spermatozoa were identified as having a functional intact plasma membrane (arrow head), normal tail spermatozoa were identified as having a non-functional intact plasma membrane.

assessed under fluorescent microscope at × 1 000 magnification and classified as intact acrosome, damaged acrosome and missing acrosome [48, 50]. The results were scored as the percentage of intact acrosome spermatozoa.

2.4.4 The functional integrity of the sperm plasma membrane

The functional integrity of the sperm plasma membrane was assessed using a short hypoosmotic swelling test (sHOST) [51]. Spermatozoa were incubated at 38ºC for 30 min, with 75 mOsm kg^{-1} of a hypoosmotic solution that consisted of 0.368% (w / v) Na-citrate and 0.675% (w / v) fructose (Merck, Germany) in distilled water. After this incubation time, 200 μL of the semen–hypoosmotic solution was fixed in 1 000 μL of hypoosmotic solution plus 5% formaldehyde (Merck, Germany), for later evaluation. A total of 200 spermatozoa were assessed under a phase contrast microscope at × 400 magnification. The coiled tail (sHOST positive) spermatozoa found after incubation had a functional intact plasma membrane (Figure 2).

2.4.5 The classification of good and poor freezability in boar spermatozoa after FT process

The boars were classified as having 'good' and 'poor' freezability according to their post-thawed progressive motility. If post-thawed progressive motility was greater than 30%, the boar sperm was

classified as good (*n* = 36 observations, 9 in each group, 3 boars). If post-thawed progressive motility was lesser than 30%, the boar sperm was classified as poor (*n* = 24 observations, 6 in each group, 2 boars).

2.5 Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, NC, USA). Normality of the data was evaluated using UNIVARIATE procedure option NORMAL PLOT. Owing to the skewed distribution of the sperm parameters, arcsine transformation was applied to all sperm parameters. Progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane (arcsine transformation) were analyzed using the General Linear Mixed Model (MIXED) procedure of the SAS. The model included the group of extender as fixed effect and boar as random effects. The statistical analysis was also performed on the basis of freezability of the boar sperm. Least-square means were obtained from each class of the factors and were compared using least significant difference. Statistically significant difference was defined as *P* < 0.05.

3 Results

In the present study, fresh semen samples were evaluated for semen quality before the FT process. On

average, the sperm concentration of the fresh semen was 354.5×10^6 spermatozoa per ml and the progressive motility was 86.0% (Table 1). The quality of FT semen is presented in Table 1. In group IV, the progressive motility and acrosome integrity were higher than group I ($P < 0.01$) (Table 1). The progressive motility of the spermatozoa in groups III and IV was higher than in group I ($P < 0.01$). There was no difference between the sperm viability and the functional integrity of the sperm plasma membrane among the groups ($P > 0.05$) (Table 1). However, the highest percentage of the sperm viability and the functional integrity of the sperm plasma membrane seemed to be observed in group IV.

The post-thawed semen qualities, that is, progressive motility, sperm viability, acrosome integrity and functional integrity of the sperm plasma membrane between good and poor freezability of boar spermatozoa are presented in Table 2. The supplementation of both DHA and *L*-cysteine (group IV) increased progressive motility by 6.2% (compared with group I, $P = 0.59$) in the good freezability semen groups ($P < 0.05$), whereas it improved by only 1.7% in the poor freezability semen groups ($P = 0.59$) (Table 2). The sperm viability in group IV increased by 8.5% compared

with group I ($P = 0.15$) in the good freezability semen groups, but decreased by 8.9% in the poor freezability semen groups ($P = 0.99$). The acrosome integrity in group IV increased by 11.7% compared with group I ($P = 0.14$) in the good freezability semen groups, whereas it improved by 6.2% in the poor freezability semen groups ($P = 0.16$). The functional integrity of sperm plasma membrane in group IV decreased by 1.5% compared with group I ($P = 0.57$) in the good freezability semen groups, whereas it decreased by 4.1% in the poor freezability semen groups ($P = 0.99$).

4 Discussion

In the present study, the supplementation of *L*-cysteine in the freezing extender improved the motility of boar spermatozoa after cryopreservation. This finding is in accordance with Bilodeau *et al.* [38] who found that the supplementation of *L*-cysteine promoted the motility of the FT bull spermatozoa. In addition, it has been shown that the supplement of *L*-cysteine in extended fresh semen significantly increases sperm viability and acrosome integrity [43, 44]. Recently, *L*-cysteine has also been shown to

Table 1. Means ± standard deviation of progressive motility (%), sperm viability (%), acrosome integrity (%) and sHost (%) of fresh sperm and frozen–thawed boar sperm between four groups ($n = 15$ ejaculates per group).

Sperm parameters	Fresh sperm	Frozen–thawed sperm			
		Group I	Group II	Group III	Group IV
Progressive motility (%)	86.0 ± 3.4	23.7 ± 7.7[a]	27.3 ± 9.2[ab]	35.3 ± 11.7[b]	36.3 ± 10.6[b]
Sperm viability (%)	85.9 ± 5.4	46.4 ± 13.3[a]	49.0 ± 14.0[a]	51.6 ± 12.8[a]	53.7 ± 12.4[a]
Acrosome integrity (%)	83.7 ± 7.3	31.9 ± 12.1[a]	37.0 ± 12.4[ab]	41.8 ± 15.6[ab]	49.1 ± 12.6[b]
sHost (%)[a]	74.8 ± 8.2	17.4 ± 9.5[a]	18.5 ± 10.1[a]	19.5 ± 8.2[a]	21.8 ± 10.7[a]

[a]sHost = functional integrity of sperm plasma membrane. Values followed by different alphabets within the same row against each parameter were significantly different ($P < 0.01$).

Table 2. Means ± standard deviation of progressive motility (%), sperm viability (%), acrosome integrity (%) and sHost (%) of frozen-thawed boar semen (good freezability and poor freezability) between four groups.

Sperm parameters	Frozen–thawed Semen							
	Poor freezability				Good freezability			
	Group I ($n = 6$)	Group II ($n = 6$)	Group III ($n = 6$)	Group IV ($n = 6$)	Group I ($n = 9$)	Group II ($n = 9$)	Group III ($n = 9$)	Group IV ($n = 9$)
Progressive motility (%)	20.0 ± 4.5[a]	21.3 ± 2.5[a]	20.0 ± 2.7[a]	21.7 ± 2.9[a]	33.8 ± 4.8[a]	35.7 ± 6.1[a]	40.5 ± 9.1[b]	40.0 ± 8.3[b]
Sperm viability (%)	45.4 ± 15.0[a]	34.6 ± 14.0[a]	40.3 ± 13.1[a]	36.5 ± 12.2[a]	49.1 ± 7.8[a]	58.2 ± 9.1[a]	56.9 ± 7.2[a]	57.6 ± 8.9[a]
Acrosome integrity (%)	30.5 ± 11.9[a]	36.7 ± 12.9[a]	28.1 ± 7.1[a]	36.7 ± 12.9[a]	38.3 ± 9.6[a]	47.3 ± 8.4[a]	48.1 ± 12.4[a]	50.0 ± 11.8[a]
sHost (%)[a]	14.2 ± 8.9[a]	12.3 ± 7.4[a]	11.3 ± 4.6[a]	10.1 ± 4.3[a]	26.3 ± 4.1[a]	26.8 ± 7.9[a]	22.1 ± 6.9[a]	24.8 ± 9.8[a]

[a]sHost = functional integrity of sperm plasma membrane. Values followed by different alphabets within the same row against each parameter were significantly different ($P < 0.01$).

Supplement of DHA-enriched hen egg yolk and *L*-cysteine
Panida Chanapiwat *et al*.

npg

605

improve sperm viability and acrosome integrity of FT ram's semen [39]. The positive effects of *L*-cysteine on many sperm parameters might be because of several factors, for example, *L*-cysteine can reactivate ROS and catalyze the detoxification of H_2O_2 and other superoxides [52]. However, the susceptibility of spermatozoa to LPO differs among species. The doses of antioxidant used for different species may be different. In the present study, 5 mmol L^{-1} of *L*-cysteine was used, because a previous study reported that a concentration of between 2.5 and 5 mmol L^{-1} of *L*-cysteine improved extended boar semen qualities [44]. In addition, the present study found that *L*-cysteine only improved motility in good freezability boars, whereas no improvement was noted in poor freezability boars. To our knowledge, this finding has never been reported before. It has been shown that there are differences in specific DNA sequences between boars that have poor and good post-thawed semen quality [53]. In addition, the mutation of ubiquitin-specific protease 26 gene on the X-chromosome has been observed in 22% of infertile men [54]. In our study, poor freezability boars might have had an abnormal DNA sequence or a mutation of some specific genes. The supplementation of *L*-cysteine may not improve the FT semen quality in these boars.

In the present study, we found that the supplement of DHA-enriched hen egg yolk alone did not improve the post-thawed semen quality. In an earlier study, Maldjian *et al*. [42] also failed to improve the post-thawed semen quality after using DHA-enriched hen egg yolk instead of normal hen egg yolk. However, they have shown that the proportion of DHA in phospholipids of spermatozoa could be increased by using DHA-enriched hen egg yolk. In the present study, the percentage of DHA in the sperm plasma membrane has not been evaluated. On the other hand, Kaeoket *et al*. [19] found that the supplementation of DHA by adding fish oil to the freezing extender successfully improved the progressive motility, viability, plasma membrane integrity and acrosome integrity of FT boar spermatozoa. Therefore, in the present study, the reason that DHA alone failed to improve the post-thawed semen quality might be because of a low concentration of DHA in the DHA-enriched hen egg yolk. Furthermore, the ability of DHA uptake of spermatozoa might differ among individual boars and breeds [55]. In the animal model, it was found that the supplement of DHA in boar feed significantly increased the progressive motility and intact acrosome of fresh semen [56, 57]. However, the supplement of DHA-enriched cod liver oil in boar diet failed to improve the freezability of boar semen, even though cod liver oil supplement increased DHA content of boar spermatozoa plasma membranes [58]. The influence of DHA on the freezing ability of boar spermatozoa is still ambiguous and remains to be further investigated. Sources and concentrations of DHA, as well as breed and the individual boar effect on DHA uptake should also be taken into consideration.

In the present study, the combination of DHA-enriched hen egg yolk and *L*-cysteine significantly increased progressive motility and intact acrosome. The effect of this combination has never been studied before. It is known that both DHA and *L*-cysteine play a major role in protecting sperm structure and function. In general, differences in the lipid composition of the sperm plasma membrane influenced the freezability of the spermatozoa. The proportion of PUFAs, especially DHA and DPA, in the plasma membrane of spermatozoa is affected by use of diluents and by freezing [42]. High levels of PUFAs in the membrane phospholipids increase membrane fluidity and flexibility [59], and subsequently improve freezability of the spermatozoa. In the present study, semen extender containing both DHA and *L*-cysteine resulted in the highest post-thawed semen qualities, especially progressive motility and acrosome integrity. Acrosome integrity is a parameter used to evaluate the functional membrane status of the spermatozoa [49]. Viable spermatozoa with an intact acrosome are able to undergo the acrosome reaction, penetrate the zona pellucida and fuse with the oocyte. The DHA and cysteine combination may protect the acrosome by enhancing fluidity and increasing ROS scavenging. However, the use of DHA alone did not produce a significant difference. This might be because of the low concentration of DHA used in the present study compared with that used by Kaeoket *et al*. [19]. To improve the post-thawed boar semen quality, the effect of concentrations of DHA and *L*-cysteine should be investigated.

Interestingly, in the present study, neither DHA nor *L*-cysteine supplementation improved the FT sperm parameters (that is, progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane) in poor freezability spermatozoa. On the other hand, DHA and *L*-cysteine did improve the FT semen qualities of good freezability spermatozoa.

This might be due to the difference of lipid composition in sperm plasma membrane, and because the ability of DHA and *L*-cysteine uptake of spermatozoa differs among boars [55]. The reason for differences in the freezability of boar spermatozoa is unknown at present. A possible explanation might be related to the genetic variation and/or abnormalities of the DNA and genes (see above) among boars [56]. It has been reported that the cryopreservation process significantly reduced intracellular glutathione (GSH) levels of boar spermatozoa [17]. Therefore, supplementation with *L*-cysteine, a precursor of intracellular GSH biosynthesis, has a beneficial effect while intracellular GSH levels are decreased.

In conclusion, the combination of *L*-cysteine and DHA-enriched hen egg yolk significantly improved the progressive motility and acrosome integrity of FT boar spermatozoa. DHA-enriched hen egg yolk alone failed to improve the quality of boar sperm after cryopreservation.

Acknowledgment

References

1 Polge C. Artificial insemination in pigs. Vet Rec 1956; 68: 62–76.

2 Larsson K, Einarsson S. Fertility of deep frozen boar spermatozoa. Acta Vet Scand 1976; 17: 43–62.

3 Almlid T, Hofmo PO. A brief review of frozen semen application under Norwegian AI service conditions. Reprod Domest Anim 1996; 31: 169–73.

4 Johnson LA. Current developments in swine semen: preservation, artificial insemination and sperm sexing. Proc 15th IPVS 1998; 1: 225–9.

5 Bailey JL, Lessard C, Jacques J, Brèque C, Dobrinski I, *et al.* Cryopreservation of boar semen and its future importance to the industry. Theriogenology 2008; 70: 1251–9.

6 Waberski D, Weitze KF, Gleumes T, Schwarz M, Willmen T, *et al.* Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. Theriogenology 1994; 42: 831–40.

7 Saravia F, Wallgren M, Nagy S, Johannison A, Rodriguez-Martinez H. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. Theriogenology 2005; 63: 1320–33.

8 Eriksson BM, Petersson H, Rodriguez-Martinez H. Field fertility with exported boar semen frozen in the new flatpack container. Theriogenology 2002; 58: 1065–79.

9 Wongtawan T, Saravia F, Wallgren M, Caballero I, Rodriguez-Martinez H. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. Theriogenology 2006; 65: 773–87.

10 Johnson LA, Weitze KF, Fiser P, Maxwell WMC. Storage of boar semen. Anim Reprod Sci 2000; 62: 142–72.

11 Eriksson BM, Rodriguez-Martinez H. Effect of freezing and thawing rate on the post-thaw viability of boar spermatozoa frozen in FlatPack and Maxi-straws. Anim Reprod Sci 2000; 63: 205–20.

12 Córdova-Izquierdo A, Oliva JH, Lleó B, García-Artiga C, Corcuera BD, *et al.* Effect of different thawing temperatures on the viability, *in vitro* fertilizing capacity and chromatin condensation of frozen boar semen packaged in 5 ml straws. Anim Reprod Sci 2006; 92: 145–54.

13 Bwanga CO, Einarsson S, Rodriguez-Martinez H. Deep freezing of boar semen packaged in plastic bags and straws. Reprod Domest Anim 1991; 26: 117–25.

14 Berger B, Fischerleitner F. On deep freezing of boar semen: investigations on the effects of different straw volumes, methods of freezing and thawing extenders. Reprod Domest Anim 1992; 27: 266–70.

15 Buranaamnuay K, Tummaruk P, Singlor J, Rodriguez-Martinez H, Techakumphu M. Effects of straw volume and Equex-STM® on boar sperm quality after cryopreservation. Reprod Domest Anim 2009; 44: 69–73.

16 Peñã FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 2003; 78: 85–98.

17 Gadea J, Selles E, Marco MA, Coy P, Matas C, *et al.* Decrease in gluthathione content in boar sperm after cryopreservation, effect of the addition of reduced glutathione to the freezing and thawing extender. Theriogenology 2004; 62: 690–701.

18 Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, *et al.* Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004; 25: 397–405.

19 Kaeoket K, Sang-urai P, Thamniyom A, Chanapiwat P, Techakumphu M. Effect of Docosahexaenoic acid (DHA) on quality of cryopreserved boar semen in different breeds. Reprod Domest Anim 2008; In press.

20 Pursel VG, Schulman LL, Johnson A. Effect of Orvus Es Paste on acrosome morphology, motility and fertility capacity of frozen thawed sperm. J Anim Sci 1978; 47: 198–201.

21 Fraser L, Strezezek J. Effect of different procedures of

Supplement of DHA-enriched hen egg yolk and *L*-cysteine
Panida Chanapiwat *et al*.

npg

607

ejaculate collection, extenders and packages on DNA integrity of boar sperm following freezing-thawing. Anim Reprod Sci 2007; 99: 317–29.

22 Eriksson BM, Vazquez JM, Martinez EA, Roca J, Lucas X, *et al*. Effects of holding time during cooling and of type of package on plasma membrane integrity, motility and *in vitro* oocyte penetration ability of frozen-thawed boar spermatozoa. Theriogenology 2001; 55: 1593–605.

23 Holt WV, Medrano A, Thurston LM, Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation insights from the cryomicroscope. Theriogenology 2005; 63: 370–82.

24 Roca J, Hernandez M, Carvajal G, Vazquez JM, Martinez EA. Factors influencing boar sperm cryosurvival. J Anim Sci 2006; 84: 2692–9.

25 Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 2000; 53: 47–58.

26 Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; 60–1: 481–92.

27 Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 2000; 1469: 197–235.

28 Johnson LA, Gerrits RJ, Young EP. The fatty acid composition of porcine spermatozoa phospholipids. Biol Reprod 1969; 1: 330–4.

29 Parks JE, Lynch DV. Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. Cryobiology 1992; 29: 255–66.

30 Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology 1977; 14: 466–70.

31 de Lamirande ED, Gagnon C. 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 1992; 13: 368–86.

32 Sikka SC, Rajasekaran M, Hellstrom WJ. Role of oxidative stress and antioxidants in male infertility. J Androl 1995; 16: 464–8.

33 Chatterjee S, De Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol Reprod Dev 2001; 60: 498–506.

34 Agarwal A, Prabakaran SA, Said TM. Prevention of oxidative stress injury to sperm. J Androl 2005; 26: 654–60.

35 Aurich JE, Schönherr U, Hoppe H, Aurich C. Effects of antioxidants on motility and membrane integrity of chilled stored stallion semen. Theriogenology 1997; 48: 185–92.

36 Ball BA, Medina V, Gravance CG, Baumbe J. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5 °C. Theriogenology 2001; 56: 577–89.

37 Beconi MT, Francia CR, Mora NG, Affranchino MA. Effect of natural antioxidants on frozen bovine semen preservation. Theriogenology 1993; 40: 841–51.

38 Bilodeau JF, Blanchette S, Gagnon C, Sirard MA.

39 Uysal O, Bucak MN. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 2007; 76: 383–90.

40 Bucak MM, Atessahin A, Varisli Ő, Yuce A, Tekin N, *et al*. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology 2007; 67: 1060–7.

41 Roca J, Rodriguez JM, Gil MA, Carvajal G, Garcia EM, *et al*. Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005; 26: 15–24.

42 Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, *et al*. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology 2005; 63: 411–21.

43 Szczesniak-Fabianczyk B, Bochenek M, Smorag Z, Silvestre MA. Effects of antioxidants added to boar semen extender on the semen survival and sperm chromatin structure. Reprod Biol 2003; 3: 81–7.

44 Funahashi H, Sano T. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. Theriogenology 2005; 63: 1605–16.

45 Westendorf PL, Richter L, Treu H. Zur Tiefgefrierung von Ebersperma. Labor- und Besamungsergebnisse mit dem Hulsenberger Pailletten-verfahren. Dtsch Tierarztl Wochenschr 1975; 82: 261–7.

46 Selles E, Gadea J, Romar R, Matas C, Ruiz S. Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. Reprod Domest Anim 2003; 38: 66–72.

47 Beardon HJ, Fuquay JW. Semen evaluation. In: Beardon HJ, Fuquay JW, editors. Applied Animal Reproduction, 4th ed. New Jersey: Prentice Hall; 1997. p158–70.

48 Axnér E, Hermansson U, Linde-Forsberg C. The effect of Equex STM paste and sperm morphology on frozen-thawed survival of cat epididymal sperm. Anim Reprod Sci 2004; 84: 179–91.

49 Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995; 53: 276–84.

50 Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, *et al*. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J Androl 1996; 17: 674–82.

51 Perez-Llano B, Lorenzo JL, Yenes P, Trejo A, Garcia-Casado P. A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology 2001; 56: 387–98.

52 Meister A. On the antioxidant effects of ascorbic acid and glutathione. Biochem Pharmacol 1992; 44: 1905–15.

53 Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers links to genes controlling boar

Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 2001; 56: 275–86.

sperm viability following cryopreservation. Biol Reprod 2002; 66: 545–54.

54 Zhang J, Qiu SD, Li SB, Zhou DX, Tian H, *et al.* Novel mutations in ubiquitin-specific protease 26 gene might cause spermatogenesis impairment and male infertility. Asian J Androl 2007; 9: 809–14.

55 Vazquez JM, Roldan ERS. Phospholipid metabolism in boar spermatozoa and role of diacylglycerol species in the de novo formation of phosphatidylcholine. Mol Reprod Dev 1997; 47: 105–12.

56 Rooke JA, Shao CC, Speake BK. Effects of feeding tuna oil on the lipid composition of pig spermatozoa and *in vitro* characteristics of semen. Reproduction 2001; 121: 315–22.

57 Strzezek J, Fraser L, Kuklinska M, Dziekonska A, Lecewicz M. Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. Reprod Biol 2004; 4: 271–87.

58 Paulenz H, Taugbøl O, Kommisrud E, Grevle IS. Effect of dietary supplementation with cod liver oil on cold shock and freezability of boar semen. Reprod Domest Anim 1999; 34: 431–5.

59 Lenzi A, Picardo M, Gandini L, Dondero F. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum Reprod Update 1996; 2: 246–56.