Original Article

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

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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.

Keywords: boar, DHA, extender, frozen semen, L-cysteine

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 $5 \times 10^9$ 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 glutathione 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 ≥ 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 15°C for 120 min and centrifuged at 800 x 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 x 10⁹ spermatozoa per mL. The diluted semen was cooled to 5°C 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 x 10⁹ 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 3°C min⁻¹ from +5°C to −5°C and thereafter 50°C min⁻¹ from −5°C to −140°C. Then the straws were plunged into liquid nitrogen (−196°C) for storage. Thawing was achieved by immersing the straws in water at 50°C 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 37°C 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 38°C 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 37°C 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 37°C 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 4°C for 30 min. After incubation, it was rinsed with cold PBS and air dried. A total of 200 spermatozoa were
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 38°C 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 $354.5 \times 10^5$ 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 freezeability 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 freezeability semen groups ($P < 0.05$), whereas it improved by only 1.7% in the poor freezeability 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 freezeability semen groups, but decreased by 8.9% in the poor freezeability 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 freezeability semen groups, whereas it improved by 6.2% in the poor freezeability 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 freezeability semen groups, whereas it decreased by 4.1% in the poor freezeability semen groups ($P = 0.99$).

### 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

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**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 (%) | 23.7 ± 7.7<sup>a</sup> | 27.3 ± 9.2<sup>b</sup> | 35.3 ± 11.7<sup>b</sup> | 36.3 ± 10.6<sup>b</sup> |
| Sperm viability (%)     | 46.4 ± 13.3<sup>b</sup> | 49.0 ± 14.0<sup>a</sup> | 51.6 ± 12.8<sup>a</sup> | 53.7 ± 12.4<sup>a</sup> |
| Acrosome integrity (%)  | 31.9 ± 12.1<sup>a</sup> | 37.0 ± 12.4<sup>b</sup> | 41.8 ± 15.6<sup>b</sup> | 49.1 ± 12.6<sup>a</sup> |
| sHost (%)<sup>ab</sup>  | 17.4 ± 9.5<sup>a</sup> | 18.5 ± 10.1<sup>a</sup> | 19.5 ± 8.2<sup>a</sup> | 21.8 ± 10.7<sup>a</sup> |

<sup>a</sup>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 freezeability and poor freezeability) between four groups.

| Sperm parameters | Poor freezeability | Good freezeability |
|------------------|-------------------|--------------------|
|                  | 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<sup>a</sup> | 21.3 ± 2.5<sup>a</sup> | 20.0 ± 2.7<sup>a</sup> | 21.7 ± 2.9<sup>a</sup> | 33.8 ± 4.8<sup>b</sup> | 35.7 ± 6.1<sup>b</sup> | 40.5 ± 9.1<sup>b</sup> | 40.0 ± 8.3<sup>b</sup> |
| Sperm viability (%)     | 45.4 ± 15.0<sup>a</sup> | 34.6 ± 14.0<sup>a</sup> | 40.3 ± 13.1<sup>a</sup> | 36.5 ± 12.2<sup>a</sup> | 49.1 ± 7.8<sup>b</sup> | 58.2 ± 9.1<sup>b</sup> | 56.9 ± 7.2<sup>b</sup> | 57.6 ± 8.9<sup>b</sup> |
| Acrosome integrity (%)  | 30.5 ± 11.9<sup>a</sup> | 36.7 ± 12.9<sup>a</sup> | 28.1 ± 7.1<sup>a</sup> | 36.7 ± 12.9<sup>a</sup> | 38.3 ± 9.6<sup>a</sup> | 47.3 ± 8.4<sup>b</sup> | 48.1 ± 12.4<sup>b</sup> | 50.0 ± 11.8<sup>a</sup> |
| sHost (%)<sup>ab</sup>  | 14.2 ± 8.9<sup>a</sup> | 12.3 ± 7.4<sup>a</sup> | 11.3 ± 4.6<sup>a</sup> | 10.1 ± 4.3<sup>a</sup> | 26.3 ± 4.1<sup>a</sup> | 26.8 ± 7.9<sup>a</sup> | 22.1 ± 6.9<sup>a</sup> | 24.8 ± 9.8<sup>a</sup> |

<sup>a</sup>sHost = functional integrity of sperm plasma membrane. Values followed by different alphabets within the same row against each parameter were significantly different ($P < 0.01$).
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$_2$O$_2$ 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.

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