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Original Article

Supplemental effect of varying *L*-cysteine concentrations on the quality of cryopreserved boar semen

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Abstract

Cryopreservation is associated with the production of reactive oxygen species, which leads to lipid peroxidation of the sperm membrane and consequently a reduction in sperm motility and decreased fertility potential. The aim of this study was to determine the optimal concentration of *L*-cysteine needed for cryopreservation of boar semen. Twelve boars provided semen of proven motility and morphology for this study. The semen was divided into four portions in which the lactose-egg yolk (LEY) extender used to resuspend the centrifuged sperm pellet was supplemented with various concentrations of *L*-cysteine to reach 0 mmol L^{-1} (group I, control), 5 mmol L^{-1} (group II), 10 mmol L^{-1} (group III) and 15 mmol L^{-1} (group IV). Semen suspensions were loaded in straws (0.5 mL) and placed in a controlled-rate freezer. After cryopreservation, frozen semen samples were thawed and investigated for progressive motility, viability using SYBR-14/EthD-1 staining and acrosome integrity using FITC-PNA/EthD-1 staining. There was a significantly higher ($P < 0.01$) percentage of progressive motility, viability and acrosomal integrity in two *L*-cysteine-supplemented groups (group II and group III) compared with the control. There was a biphasic effect of *L*-cysteine, with the highest percentage of progressive motility, viability and acrosomal integrity in group III. In conclusion, 5 or 10 mmol L^{-1} was the optimum concentration of *L*-cysteine to be added to the LEY extender for improving the quality of frozen–thawed boar semen.

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1 Introduction

The technology for cryopreservation of boar semen has been developed for preservation of genetically superior boars and the transport of genetic lines across countries. The detrimental effect of cryopreservation on spermatozoa has been reported, e.g. cold-shock, osmotic stress and oxidative stress from reactive oxygen species (ROS), which impair normal sperm function and fertilizing potential [1, 2]. Weir and Robaire [3] reported that the seminal plasma consists of enzymatic antioxidants, i.e. glutathione reductase, glutathione peroxidases, superoxide dismutase and catalase, which have the ability to reduce lipid-peroxidation-producing free radicals and thus to protect spermatozoa from ROS [2, 4, 5]. During cryopreservation, it has been demonstrated that some steps of cryopreservation, such as discarding of seminal plasma by centrifugation and reduction in temperature, result in reduced quality and quantity of antioxidants and an increase in the quantity of ROS contained in the semen [6]. This ROS causes

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lipid peroxidation of the sperm plasma membrane, which has detrimental effects (i.e. decreased progressive motility and acrosomal integrity, increased mid-piece abnormalities and inhibited sperm–oocyte fusion) on spermatozoa during cryopreservation [7–10].

During the past few years, many studies have been made on supplementation with various antioxidants (e.g. vitamin E, glutathione, taurine) in the freezing extenders of frozen boar semen in an attempt to minimize the detrimental effect of ROS, which occurs during the freezing process [11–14]. Funahashi and Sano [15] demonstrated that a supplement of *L*-cysteine (5 mmol L^{-1}) could improve the viability and progressive motility of fresh extended boar semen. This effect was also demonstrated for frozen bovine semen [6]. *L*-cysteine also improved the survival of spermatozoa and sperm chromatin structure in fresh chilled boar semen at 15ºC [16]. For frozen dog semen, Michael *et al*. [17] reported that a supplement of *L*-cysteine increased viability and rapid steady-forward movement (RSF movement). It can be hypothesized that the concentration of *L*-cysteine (5 mmol L^{-1}) used for fresh boar semen preservation reported by Funahashi and Sano [15] may differ from the concentration that is needed for cryopreservation of boar semen. No study has reported on the optimal concentration of *L*-cysteine required for cryopreservation of boar semen. Therefore, the objective of this study was to investigate the effect of additional *L*-cysteine at different concentrations (i.e. 5, 10, 15 mmol L^{-1}) on the qualities (i.e. progressive motility, viability and acrosome integrity) of frozen–thawed semen in Landrace, Duroc and Large white boars so as to find the optimal concentration of *L*-cysteine required for cryopreservation.

2 Materials and methods

The research proposal of this project was approved by the Faculty of Veterinary Science, Animal Care and Use Committee (FVS-ACUC)-Protocol No. 2007-03 (Nakorn-pathom, Thailand).

2.1 Animals

Three breeds of boar, i.e. Duroc (n = 4), Landrace (n = 4) and Large white (n = 4), aged between 1 and 3 years, having proven fertility and from the same commercial herd were included. The boars were housed in individual pens in an open-house system from the same farm. Water was provided *ad libitum* via a water nipple. A corn–soyabean–fishmeal-based feed (15%–16% protein) was given twice a day (approximately 3 kg per day).

2.2 Preparation of boar spermatozoa

Semen samples from each boar (one ejaculate from each boar) were collected using the gloved-hand technique [18, 19]. During collection the semen was filtered through gauze, and only sperm-rich fractions were collected. Within 30 min after collection, semen volume, pH value (determined using a pH paper, Universalindikation [pH 0–14], Merck, Darmstadt, Germany) and the progressive motility of spermatozoa (i.e. only spermatozoa that move forward in a straight-line pattern) determined by a phase-contrast microscope (CX31; Olympus, New York, NY, USA) were evaluated. Semen samples of 1 mL were examined after collection into Eppendorf tubes for further analysis of concentration, in a Neubauer haemocytometer (Improved Neubauer's chamber, BOECO, Humburg, Germany), sperm viability, by the living-cell nucleic-acid stain SYBR-14 (Fertilight; Sperm Viability Kit, Molecular Probes Europe BV, Leiden, The Netherlands), sperm acrosomal integrity, by fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining, and sperm head and tail morphology, by Williams staining and formal-saline solution [19, 20]. The remaining semen was diluted with (1:1 [v/v]) Modena extender (Extender I) (Swine Genetics International Ltd., Cambridge, IA, USA) and transported by cell incubator (Micom control system 20Q, Continental Plastic CORP, Delevan, WI, USA) at 15ºC to the semen laboratory. Only ejaculates with motility of ≥ 70% and ≥ 80% morphologically normal forms were used for cryopreservation.

2.3 Semen freezing process

All semen samples were frozen in a controlled-rate freezer (Icecube 14s; Sylab, Purkersdorf, Austria).

After collection and evaluation, the semen was diluted (1:1 v/v) with Modena extender (Extender I) (Swine Genetics International Ltd.). Diluted semen was transferred to 50 mL centrifuge tubes, cooled at 15ºC for 120 min and later centrifuged at 800 × g at 15ºC for 10 min (Hettich Rotanta 460R; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant was discarded and the sperm pellet was re-suspended (about 1–2:1) with Extender II (80 mL of 11% lactose and 20 mL egg yolk) to a concentration of 1.5×10^9 spermatozoa per mL [8]. To

prepare Extender II (plus semen) supplemented with *L*-cysteine (Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland), stock solutions of *L*-cysteine were prepared as follows to provide concentrations of 5, 10 and 15 mmol L^{-1}. Stock solution A contained 302.9 mg per 5 mL distilled water; stock solution B contained 605.8 mg per 5 mL distilled water; and stock solution C contained 908.7 mg per 5 mL distilled water. Each mL of Extender II (plus semen) was mixed with 0.6058 mg (10 μL from stock solution A), 1.2116 mg (10 μL from stock solution B) or 1.8174 mg (10 μL from stock solution C) of *L*-cysteine to result in semen supplemented with 5, 10, 15 mmol L^{-1} of *L*-cysteine in extender II [20]. Thus, four groups of semen were prepared as follows:

Group I not supplemented with *L*-cysteine (control).
Group II supplemented with *L*-cysteine 5 mmol L^{-1}.
Group III supplemented with *L*-cysteine 10 mmol L^{-1}.
Group IV supplemented with *L*-cysteine 15 mmol L^{-1}.

The diluted semen was incubated at 5ºC for 90 min [8]. The four groups of semen were each mixed with a half volume of Extender III (89.5% lactose-egg yolk [LEY] extender with 9% [v/v] glycerol and 1.5% [v/v] Equex-STM; Equex-STM; Nova Chemical Sale Inc., Scituate, MA, USA). The final sperm concentration was 1.0×10^9 spermatozoa per mL. The sperm suspensions were loaded into 0.5 mL polyvinyl chloride medium-straws (Bio-Vet, Fleurance, France) and sealed with plasticine. All straws were placed horizontally on a rack and put into a chamber of the controlled-rate freezer set to +5ºC [19–22]. The cooling/freezing rate was as follows: 3ºC/min from +5ºC to –5ºC and 1 min of holding time and thereafter 50ºC/min from –5ºC to –140ºC. Then the straws were immediately plunged into liquid nitrogen (–196ºC) for storage and further analysis. Thawing of straws was carried out in a thermos flask at 50ºC for 12 s [19]. After thawing, the samples were diluted (1:4) with Modena extender in a test tube and incubated in a water bath at 37ºC for 15 min before evaluation.

2.4 Evaluation of spermatozoa

2.4.1 Progressive sperm motility

Progressive sperm motility was subjectively evaluated at 37ºC in a phase contrast microscope at × 100 (10 × 10) and × 400 (10 × 40) magnification [23]. Visual estimation was done by the same person, who was unaware of the treatments. Progressive motility was expressed as the percentage of motile sperm cells.

2.4.2 Assessment of sperm viability

The viability was evaluated with SYBR-14/ Ethidiumhomodimer-1 (EthD-1; Fertilight, Sperm Viability Kit, Molecular Probes). Ten μL of diluted spermatozoa was mixed with 2.7 μL of the working solution of SYBR-14 and 10 μL of EthD-1. After incubation at 37ºC for 20 min, a total of 200 spermatozoa were assessed (× 400) in a fluorescence microscope (Carl Zeiss Inc., Axioskop 40, Oberkochen, Germany). The nuclei of spermatozoa with intact plasma membranes stained green with SYBR-14, while those with damaged membranes stained red with EthD-1 [19]. Spermatozoa were classified into three types: "viable"—having intact plasma membranes (stained green); "damaged"—having damaged plasma membranes but an intact acrosome (stained red and green); and "dead"—having both plasma membranes and acrosomal damage stained with red. The results were recorded as the percentage of non-viable (damaged and dead spermatozoa) and viable spermatozoa.

2.4.3 Assessment of acrosomal integrity

The integrity of the sperm acrosome was evaluated using fluorescein isothiocyanate-labelled peanut (*A. Hypogaea*) agglutinin (FITC-PNA) staining [8, 24–27]. Ten μL of diluted semen with 140 μL phosphate buffered saline (PBS) was mixed with 10 μL of EthD-1 and incubated at 37ºC for 15 min. Then 5 μL of the suspension was smeared on glass slides and fixed with 95% ethanol for 30 s and air-dried. In the next step, 50 μL FITC-PNA (FITC-PNA diluted with PBS [1:10, v/v]) was spread over the slide and incubated in a moist chamber at 4ºC for 30 min. After incubation, the slide was rinsed with cold PBS and air-dried. A total of 100 spermatozoa were assessed in a fluorescence microscope at ×1 000 magnification, and classified as those with intact acrosomes and those with non-intact acrosomes (reacted and loose acrosome) [26].

2.5 Statistical analysis

Data were analyzed by using the general linear model (SPSS 13.0; SPSS Inc, Chicago, IL, USA) and expressed as mean ± SD. The specific sperm treatment and breeds of boars were modelled according to the Factorial Experiments in Randomized Complete block Design (RCBD) and analysed with the PROC GLM. When the GLM revealed a significant effect, the mean values were compared by Scheffe's test with $P < 0.05$.

3 Results

3.1 Fresh semen analysis

Fresh semen was analyzed for progressive motility, morphology, viability, acrosomal integrity and head morphology. No significant differences in semen qualities were found among breeds (Table 1).

3.2 Post-thawed semen analysis

No effect of breed on frozen–thawed semen quality was seen in the present results.

A higher percentage of progressive motility was found in two treatment groups (groups II and III) (Figure 1) than in the other groups ($P < 0.01$).

A higher percentage of viable spermatozoa was found in two treatment groups (groups II and III) (Figure 2) than in the other groups ($P < 0.01$). The highest percentage of viable spermatozoa was in group III (Figure 2).

With regard to acrosomal integrity in live spermatozoa, the percentage of cells with intact acrosomes was significantly higher in the treatment groups than in the control group ($P < 0.01$). The highest percentage of intact acrosome was in group III (Figure 3).

4 Discussion

None of the fresh semen quality parameters, i.e. sperm morphology, progressive motility, viability and acrosomal integrity, were significantly different among breeds of boar, indicating the uniformity of samples used in the study. In the present study, it is clearly shown that adding *L*-cysteine at a concentration of 5 or 10 mmol L^{-1} in the freezing Extender II produces better results in terms of progressive motility, viability and acrosomal integrity compared with the other groups. To our knowledge, this is the first study showing

Table 1. Percentage (mean ± SD) of head and tail morphology, progressive motility, viability and acrosome integrity in extended fresh boar semen in different breeds.

Parameters	Breeds			
	Duroc ($n = 4$)	Landrace ($n = 4$)	Large white ($n = 4$)	Overall significance
Progressive motility (%)	91.3 ± 2.5	90.0 ± 4.1	90.0 ± 0.0	NS
Normal morphology (%)	89.0 ± 4.3	93.0 ± 3.5	80.8 ± 5.5	NS
Viability (%)	88.9 ± 10.0	83.9 ± 13.1	87.3 ± 8.2	NS
Acrosome integrity in live spermatozoa (%)	74.5 ± 12.5	78.0 ± 10.7	82.0 ± 2.2	NS

Abbreviation: NS; no significant differences ($P > 0.05$).

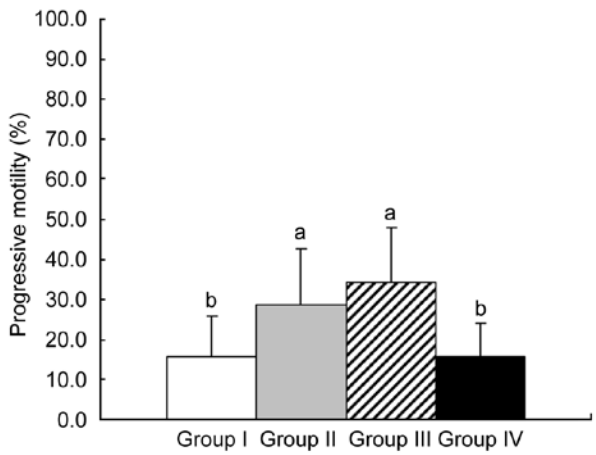

Figure 1. Progressive motility of spermatozoa in frozen–thawed semen in different concentrations of *L*-cysteine: group I (control), group II (5 mmol L^{-1}), group III (10 mmol L^{-1}), group IV (15 mmol L^{-1}) presented as bars (mean ± SD, $N = 12$). Bars marked by different letters are significantly different ($P < 0.01$).

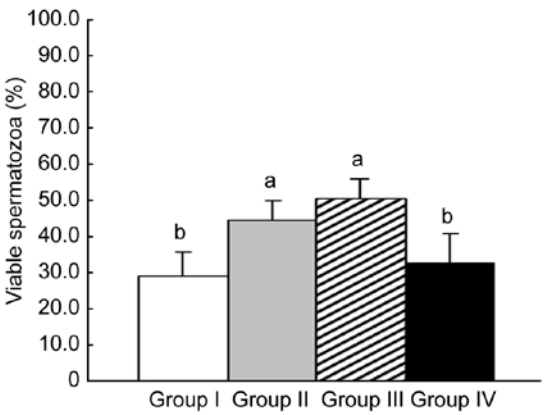

Figure 2. Viability of spermatozoa in frozen–thawed semen in different concentrations of *L*-cysteine: group I (control), group II (5 mmol L^{-1}), group III (10 mmol L^{-1}), group IV (15 mmol L^{-1}) presented as bars (mean ± SD, $N = 12$). Bars marked by different letters are significantly different ($P < 0.01$).

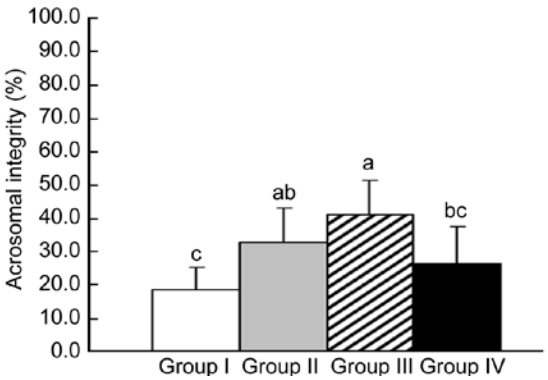

Figure 3. Acrosomal integrity of spermatozoa in frozen–thawed semen in different concentrations of cysteine: group I (control), group II (5 mmol L^{-1}), group III (10 mmol L^{-1}), group IV (15 mmol L^{-1}) presented as bars (mean ± SD, $N = 12$). Bars marked by different letters are significantly different ($P < 0.01$).

the optimum concentration of *L*-cysteine in LEY extender (Extender II) needed to produce superior-quality frozen–thawed boar semen. It might be that spermatozoa absorbed and utilized *L*-cysteine from the media to neutralize ROS, which, in turn, safeguarded their plasma membrane and inner organelles [28].

The results in the present study are in agreement with those of Bilodeau *et al*. [6] in that a supplement of *L*-cysteine could improve progressive motility in frozen–thawed bull semen improve the quality of frozen canine semen [17] and also improve sperm survival and sperm chromatin structure [16]. The improvement in the qualities of frozen–thawed boar semen found in the present study, might be explained by *L*-cysteine being a precursor of intracellular glutathione [6], whose level is decreased during the cryopreservation process, and subsequently resulting in a reduction of fertilization potential of frozen boar spermatozoa [29]. In addition, *L*-cysteine has the ability to pass into cells rapidly, and to be transformed into taurine, which, combined with a fatty acid in the plasma membrane, is transformed to acyl-taurine, which can improve surfactant properties and osmoregulation of the sperm membrane [24, 28]. Furthermore, *L*-cysteine also has the ability to reduce lipid peroxidation that produces free radicals, and thus protect spermatozoa from ROS [2, 4, 5]. Besides being active as a glutathione precursor, *L*-cysteine can also account for a protective effect in the extracellular milieu, primarily due to its nucleophilic and antioxidant properties [30].

In the present study, it is shown that the qualities of frozen–thawed boar semen depend on the concentration of *L*-cysteine. Nevertheless, too high concentration of *L*-cysteine may not be an advantageous if spermatozoa have limiting *L*-cysteine uptake [31, 32], as shown by a study on the supplementation of DHA (fish oil) in the freezing extender for cryopreservation of boar semen [19] and of *L*-cysteine in fresh extended boar semen [15].

In conclusion, addition to the LEY extender (Extender II) of *L*-cysteine at a concentration of 5 or 10 mmol/L is the optimum concentration of *L*-cysteine for improving the quality of frozen–thawed boar semen (i.e. increased progressive motility, viability and acrosome integrity).

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