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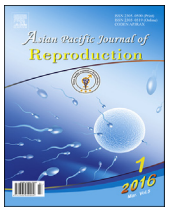

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Milk supplements in a glycerol free trehalose freezing extender enhanced cryosurvival of boar spermatozoa

Rukmali Athurupana[1], Hiroaki Funahashi[1,2*]

[1]*Department of Animal Science, Graduate School of Environmental and Life Science, Okayama University, Okayama, 700-8530, Japan*

[2]*Department of Animal Science, Faculty of Agriculture, Okayama University, Okayama, 700-8530, Japan*

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ABSTRACT

Objective: To evaluate the effect of skim milk and/or coconut milk in a glycerol-free trehalose extender to improve cryosurvival of boar spermatozoa.

Methods: Sperm samples were diluted in an egg-yolk-based freezing extender containing 100 mM trehalose and 0.25% Equex STM supplemented with coconut milk or/and skim milk at 2% or 5% (w/v). Spermatozoa were cryopreserved in 0.5 mL straws and thawed by a rapid transient method (at 70 °C for 8 s) followed with a stabilizing procedure at 39 °C. Thawed samples were analyzed for motility, viability, high mitochondrial membrane potential (HMMP), and acrosome damage.

Results: Even on the presence of egg yolk, motility, HMMP and viability were significantly higher in extender supplemented with 2% skim milk than controls without skim milk ($P < 0.05$). Post-thaw viability significantly improved with the addition of 2% skim milk plus 2% coconut milk as well ($P < 0.05$). Acrosome damage was considerably lower when the extender was supplemented with 2% coconut milk ($P < 0.05$), whereas the benefit was masked in the presence of 2% skim milk.

Conclusion: 2% skim milk can be used as supplements for a glycerol-free trehalose and egg yolk-based extender to improve post-thaw survival of boar spermatozoa, whereas 2% coconut milk has an effect to protect boar spermatozoa from acrosome damage.

1. Introduction

Sperm cryopreservation is the most efficient method for storing boar spermatozoa for a long period, even though their fertilizing ability is still lower than that of fresh or liquid-preserved semen [1]. During the past few years, substantial progress has been made regarding cryopreservation techniques for boar spermatozoa [2]. Adjustment of cooling and re-warming rates to biophysical properties of boar spermatozoa, new sperm package systems and the achievement of accurately consistent freezing of large numbers of samples using programmable freezers have contributed to post-thaw survival rates above 50%, a threshold similar to that used for bull AI-semen [3]. However, these promising results are overshadowed by low conception rates and smaller litters after artificial insemination, limiting the commercial use of cryopreserved boar spermatozoa to a sub-optimal level [4].

Boar spermatozoa are not easy to cryopreserve due to several reasons. Differences in the sperm freezability have also been reported to exist between breeds, within and between boars, between fractions coming from the same ejaculate and even between the seasons [5–8]. Besides, it is well known that sperm from species with low levels of cholesterol in their sperm membranes, such as boar, have decreased tolerances to cold shock, as compared with sperm from species with high levels of cholesterol, like human [9]. Researchers constantly experiment to develop more practical and less-expensive methods to improve cryosurvival of spermatozoa especially from low freezability boars.

In our recent studies, we have demonstrated that non-permeating sugar trehalose can maintain motility, viability, acrosome integrity, HMMP and *in vitro* penetrability, when

*Corresponding author: Hiroaki Funahashi, Department of Animal Science, Graduate School of Environmental and Life Science, Okayama University, 1-1-1 Tsushima-Naka, Kita-Ku, Okayama 700-8530 Japan.
Tel/Fax: +81 86 251 8329
E-mails: hirofun@okayama-u.ac.jp, rukmali_athurupana@yahoo.com

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boar spermatozoa were frozen in a glycerol-free freezing extender [10] and thawed after rapid transient thawing at 70 °C for 8 s followed by a stabilizing procedure at 39 °C for 52 s [11].

On the other hand, various milk based extenders have also been proven to be effective for bovine [12,13], dog [14,15], ram [16], buck [17] and equine [18,19] spermatozoa. Most studies have been carried out using whole milk, skim milk or soy milk [12,20] which the western world is mush aware of, but less attention has been paid for coconut milk, which is rich in antioxidants, fat, proteins, minerals and carbohydrates [21,22]. Therefore, the current study was undertaken to examine the effect of skim milk and coconut milk on the cryosurvival of spermatozoa in our glycerol-free trehalose freezing medium using rapid transient thawing method.

2. Materials and methods

2.1. Chemicals and extenders

Unless specified, all the chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan). The basic diluents, modified Modena Solution [23], was composed of 152.61 mM glucose, 23.46 mM sodium citrate $2H_2O$, 11.9 mM $NaHCO_3$, 6.99 mM EDTA-2Na.$2H_2O$, 46.66 mM TRIS, 15.10 mM citric acid and 10 mg/mL gentamicin. Egg yolk based extender (20% hen's egg yolk in mMS) was used as the cooling extender. Freezing extender consisted of cooling extender supplemented with 0.25% Equex STM™ (Nova chemical sales, Inc, Scituate, MA, USA), 100 mM trehalose (Hayashibara Co. Ltd, Okayama, Japan) and skim milk (S) and/or coconut milk (C). Percentages (w/v) of milk supplements and combinations in trehalose extender are as follows: T; freezing extender without milk supplements (Control), TS2; 2% skim milk, TC2; 2% coconut milk, TCS2; 2% coconut and 2% skim milk, TS5; 5% skim milk, TC5; 5% coconut milk, TCS5; 5% coconut and 5% skim milk in trehalose extenders. Stock solution (10%, w/v) of coconut milk was prepared by dissolving 1 g of coconut milk powder (MAGGI coconut milk powder, Nestle, Colombo, Sri Lanka) in 10 mL of prewarmed mMS. It was filtered using a piece of cheesecloth and centrifuged at 700 ×*g* for 15 min at room temperature. Coconut milk was carefully drawn off below the upper oily layer using a new needle and syringe to a new tube. Stock solution (10%, w/v) of skim milk was prepared by dissolving 1 g of skim milk powder (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in 10 mL of prewarmed mMS. It was centrifuged at 700 ×*g* for 15 min at room temperature. Supernatant of the skim milk solution was transferred to a new falcon tube. Both solutions were stored at 15 °C for further use.

2.2. Semen collection and processing

Semen samples were collected from three individual Berkshire boars (1–3 years old) with excellent fertility scores (supplied by a local AI center). At least three ejaculates were obtained from each boar, and collection was done once a week. The sperm-rich fraction (SRF) from individual ejaculates was collected into a prewarmed tube by gloved-hand technique. Two diluted samples were made by adding SRF to mMS (1:4) before transportation to the laboratory. The semen samples were kept in a Styrofoam box with warm packs (39 °C) and transported within 1.5 h. At the laboratory, the samples were assessed for sperm concentration (hemocytometer), viability (SYBR/propidium iodide) and motility (CASA). Sperm samples with >70% of motility and viability were used in the current experiments. SRF was centrifuged at 800 ×*g* for 5 min at room temperature and 10 mL of the supernatant was diluted in 40 mL of mMS to obtain 20% (v/v) seminal plasma. Previously diluted semen samples were centrifuged at 450 ×*g* for 5 min at room temperature and the sperm pellet was diluted with 20% seminal plasma to adjust the concentration to 1×10^8 cells/mL. Then, it was cooled to 15 °C in 4 h using a thermo block (ThermoStat plus, Eppendorf, Hamburg, Germany). After incubation at 15 °C overnight, sperm sample was washed 3 times with mMS by centrifugation at 620 ×*g* for 5 min at 15 °C. Then the concentration of the sperm suspension was readjusted to 1×10^9 cells/mL with mMS before cryopreservation.

2.3. Cryopreservation of spermatozoa

Sperm samples were suspended in the cooling extender (1:4) at 15 °C and cooled down to 5 °C over the course of 2 h (ThermoStat plus, Eppendorf, Hamburg, Germany). Then they were resuspended in the freezing extender (1:1) and loaded into precooled 0.5-mL straws (Fujihara Industry Co. Ltd., Tokyo, Japan) while keeping them on ice. Other end of the straw was sealed using a heat-sealer. The straws were frozen by keeping them 4.5 cm above the level of a liquid nitrogen bath (N_2 vapor) at approximately −160 °C for 15 min. Finally, the straws were plunged into liquid nitrogen (−196 °C) and stored for 2–3 days until thawing.

2.4. Thawing of frozen spermatozoa

Straws were thawed rapidly and transiently at 70 °C for 8 s and then rapidly maintained at 39 °C for 52 s [11]. Spermatozoa were diluted in prewarmed (39 °C) mMS (1:2) and washed once by centrifugation (700 ×*g*, 3 min, 39 °C). Each pellet was resuspended in 1 mL of mMS and post-thaw evaluations were performed after incubating at 39 °C for 5 min.

2.5. Evaluation of post-thaw spermatozoa quality

The percentage of total motile spermatozoa was determined using a computer-assisted semen analysis system (CASA, with the Sperm Motility Analysis System software, Digital Image Technology, Tokyo, Japan) with 60 frames per second. For each sample, three subsamples were analyzed, and 2 μL of each subsample was placed on an objective micrometer (Fujihira Industry Co., Ltd., Tokyo, Japan) and a minimum of 300 sperms per subsample were analyzed.

Viability, HMMP and acrosome damage was evaluated by fluorescence multiple staining procedure using PI/JC-1/FITC-PNA. Briefly, 3 μL of PI (1 mg/mL), 30 μL of FITC-PNA (200 μg/mL in PBS) and 2 μL of JC-1 (153 μm) were added to 150 μL aliquot of spermatozoa. Next, samples were incubated at 39 °C for 8 min in the dark. Then, 8 μL of the mixture was