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Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 °C

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ABSTRACT

Boar semen is occasionally transferred to different locations in liquid form at 15 °C for cryopreservation. However, the use of frozen boar semen is limited due to the high susceptibility of boar sperm to cold shock. The aim of this study was to help improve the quality of frozen boar semen by determining the changes in sperm membrane and ROS during the cryopreservation processes of 15 °C-stored boar semen. Semen was collected from ten Duroc boars and transferred to our laboratory in liquid form stored at 15 °C. After cooling to 5 °C and freezing–thawing, conventional sperm parameters (total motility, progressive motility, and normal morphology), plasma membrane integrity, acrosomal membrane status, and intracellular ROS were evaluated. Sperm function, as assessed by conventional parameters, was unaffected by cooling but was decreased by freezing–thawing ($P < 0.05$). However, the cooling and freezing–thawing processes led to damages in the sperm plasma membrane, and the cooling process caused increase in mean PNA (peanut agglutinin)-fluorescence intensity in viable acrosome-intact sperm ($P < 0.05$). In ROS evaluation, the cooling process decreased intracellular $^{\bullet}O_2$ and H_2O_2 in viable sperm ($P < 0.05$), while the freezing–thawing process increased intracellular H_2O_2 ($P < 0.05$) without change in intracellular $^{\bullet}O_2$ in viable sperm. Our results suggest that, in liquid boar semen stored at 15 °C, cooling may be primarily responsible for the destabilization of sperm membranes in viable sperm, while freezing–thawing may induce reductions in sperm function with increase in membrane damage and H_2O_2.

1. Introduction

Boar semen is occasionally shipped to different facilities in liquid form at 15 °C for freezing and long-term storage. The use of frozen boar semen is highly desirable for maintaining the germplasm representing important economic traits, preserving genetic diversity, providing for more efficient breeding, and controlling the transmission of pathogens (Guthrie and Welch, 2005). Despite utilization of long-term semen storage, the use of frozen boar semen is not as efficient as liquid semen due to the high susceptibility of boar sperm to damage during cryopreservation (Grossfeld et al., 2008). Therefore, it is necessary to define the changes in sperm upon cryopreservation of liquid-preserved boar semen in order to develop appropriate semen cryopreservation methods and to improve the quality of frozen semen.

Boar sperm are very susceptible to cold shock when cooled below 15 °C (Watson, 2000). As temperature declines, there is an inevitable reduction in the proportion of sperm that maintain normal membrane integrity, ultrastructure, and biochemical components (Johnson et al., 2000). The sperm plasma membrane is the primary site of cold-induced damage (Bailey et al., 2008), and it is likely

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that these membrane stresses are related to phase changes in lipids and an altered functional state of the membrane (Watson, 2000). It is well known that a major phase change occurs at from 5 to 15 °C (Drobnis et al., 1993), which may be the sensitive temperature range for temperature-dependent injury.

It is now accepted that cryopreservation induces the formation of reactive oxygen species (ROS) in animals (Bilodeau et al., 2000; Kim et al., 2010), and cryoinjury may be induced by ROS activity generated during sperm processing (Bailey et al., 2000). In particular, boar sperm is sensitive to peroxidative damage due to its high content of polyunsaturated fatty acids (Buhr et al., 1994; Cerolini et al., 2000; Parks and Graham, 1992; White, 1993) that serve as preferred substrates for ROS generation in membranes (Brouwers et al., 2005). However, little is known about the influence of ROS on the cryopreservation of boar sperm (Awda et al., 2009; Guthrie and Welch, 2006).

Therefore, the objective of this study was to assess changes in membrane integrity and ROS during cryopreservation (cooling and freezing–thawing) of boar sperm stored at 15 °C, in particular, focusing on changes in membrane and ROS in viable sperm.

2. Materials and methods

2.1. Collection and dilution of semen

One ejaculation each from ten mature Duroc boars was collected weekly using the gloved-hand method. Sperm rich fractions were extended (1:1 [v/v]) in Beltsville Thawing Solution (BTS; 37 mg/mL glucose, 1.25 mg/mL EDTA, 6 mg/mL sodium citrate, 1.25 mg/mL sodium bicarbonate, 0.75 mg/mL potassium chloride, 0.6 mg/mL penicillin and 1 mg/mL streptomycin) (Pursel and Johnson, 1975). After collection, the sperm concentration (using a Neubauer haemocytometer), motility, and morphology were evaluated under light microscopy, and only ejaculates with $\geq 1 \times 10^8$/mL sperm concentration, ≥70% motile sperm and ≥80% normal morphology were used. Immediately after evaluation, the diluted sperm-rich fractions were slowly cooled to 15 °C in 3 h and cooled semen was transferred to the laboratory within 24 h at 15 °C.

2.2. Cryopreservation and thawing of semen

Semen was processed according to the straw freezing procedure (Almlid and Johnson, 1988; Cordova et al., 2001; Guthrie and Welch, 2006). Briefly, semen diluted in BTS was centrifuged at 800 × *g* for 10 min at 15 °C. The supernatant was then removed, and the semen pellet was resuspended with lactose-egg yolk (LEY) extender (80 mL of 11% lactose solution and 20 mL of egg yolk, pH 6.2) to a concentration of 1.5×10^8/mL. After cooling to 5 °C for 90 min, two parts LEY-extended semen were mixed with one part LEY-glycerol-Orvus-ES-Paste (LEYGO) extender (89.5% [v/v] LEY extender, 9% [v/v] glycerol, and 1.5% [v/v] Equex STM [Nova Chemical Sales, Scituate Inc., MA, USA], pH 6.2) to a final freezing concentration of 1×10^8/mL. The diluted and cooled semen was then loaded into 0.5-mL French straws (IMV, L'Aigle, France) and placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min. After then, the straws were plunged and stored in the liquid nitrogen. Thawing was achieved by immersing the straws in a water bath at 37 °C for 20 s (Carvajal et al., 2004) and thawed sperm suspensions were diluted with BTS (1:2, v/v) at 37 °C. Thawed sperm suspension was held in a water bath for 30 min, and analyzed with sperm held at 15 °C and 5 °C, respectively, during cryopreservation. Changes in sperm function due to cooling and freezing–thawing were described through comparison of the experimental groups (cooling process: 15 °C-stored vs. 5 °C-cooled semen; freezing–thawing process: 5 °C-cooled vs. frozen-thawed semen).

2.3. Sperm evaluation

2.3.1. Conventional sperm parameters

The percentage of total motile sperm and progressively motile sperm (sperm showing rapid steady forward [RSF] movement) was estimated under microscopic examination (Rota et al., 1995). For evaluation of motility, 10 μL of semen was placed on a slide and cover-slipped. The percentage of motile and progressively motile sperm was then determined by observing a minimum of 300 sperm in at least six different fields under a bright field microscope at 400× magnification. The mean of six successive estimations was recorded as the final motility score. The morphology of sperm was evaluated using the Diff-Quik kit (International Reagents Corp., Kobe, Japan). Briefly, a drop of semen on a glass slide was placed and allowed to air-dry. The slide was then dipped in the first and second solutions seven to ten times and in the final solution ten to 15 times. Lastly, at least 200 sperm were evaluated under light microscopy at 1000× magnification, according to area which morphology occur (head, acrosome, midpiece, and tail).

2.3.2. Sperm plasma-membrane integrity

Sperm plasma-membrane integrity was assessed using a 6-carboxyfluoresceindiacetate (6-CFDA; Sigma–Aldrich, St. Louis, MO, USA)/propidium iodide (PI; Sigma–Aldrich) fluorescent staining technique (Ricci et al., 2002). Briefly, 500 μL of semen sample (1×10^6 sperm/mL) was stained with 6-CFDA (5 μL of a 1 μg/mL stock solution in DMSO) and PI (5 μL of a 0.1 mg/mL stock solution in water). Samples were then mixed, incubated at 37 °C for 15 min, and analyzed using flow cytometry. Analyzed sperm were classified as having either an intact plasma-membrane (CFDA+/PI−) or a damaged plasma-membrane (CFDA−/PI+).

2.3.3. Sperm acrosomal-membrane status

Sperm acrosomal-membrane status was evaluated using a fluorescein isothiocyanate-peanut agglutinin (FITC-PNA; Sigma–Aldrich)/PI fluorescent staining technique with a slightly modified version (de Mercado et al., 2009; Nagy et al., 2003). Briefly, 500 μL of semen sample (1×10^6 sperm/mL) was stained with FITC-peanut agglutinin (FITC-PNA, 5 μL of a 0.1 mg/mL stock solution in DMSO; Sigma–Aldrich) and PI (5 μL of a 0.1 mg/mL stock solution in water). Samples were then mixed and incubated at 37 °C for 10 min. After remixing just before analysis, sam-