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Post-thawing quality of ram spermatozoa is impaired by inclusion of boar seminal plasma in the freezing extender

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ABSTRACT

Addition of seminal plasma (SP) to freezing extenders may help reducing post-thawing sperm cryoinjuries. This study evaluated the effects of adding heterologous (boar) SP to the freezing extender on ram sperm quality after thawing. Protein content was quantified in pooled SP samples from rams and boars. Five ejaculates from four rams were frozen in a Tris-egg yolk-glycerol extender including: no (control), ram and boar SP (both at 20% of the volume, with 48 μg protein/mL). After thawing, total and progressive motility for sperm treated with SP were lower than that of the control ($P < 0.05$). For spermatozoa with intact acrosome, those in contact with boar SP had the lowest membrane integrity ($P < 0.05$), but for spermatozoa with damaged acrosome, the lowest membrane integrity occurred for those in contact with ram SP ($P < 0.05$). Greater percent of high post-thawing mitochondrial membrane potential occurred in the control than in contact with both SP ($P > 0.05$). Apoptotic-like changes were similar among treatments ($P > 0.05$), but there were fewer intact spermatozoa extended with boar SP than in the control ($P < 0.01$). Contact with extenders including boar SP harmed ram sperm quality after thawing. Post-thawing motility was decreased for ram sperm extended in contact with both homologous and heterologous SP.

1. Introduction

During ejaculation, spermatozoa are in contact with many factors contained in the seminal plasma (SP) that are secreted by the epididymis and the accessory sex glands. Spermadesins are SP proteins that bind to spermatozoa's membranes, forming protein-coating layers that act on keeping membrane stability and preventing capacitation (Topfer-Petersen et al., 1998; Manaskova and Jonakova, 2008).

Sequential exposure of spermatozoa to cooling, freezing and thawing induces structural and functional damages in their organelles (Watson, 2000). Relevant cryoinjuries on spermatozoa's membranes occur due to thermal, mechanical and osmotic stresses, leading to events that resemble processes that occur during capacitation (Bailey et al., 2000), impairing sperm viability after thawing (Aisen et al., 2005).

Post-thawing damages attributed to cold shock may be repaired by contact of ram sperm with homologous SP (Barrios et al., 2000; Bernardini et al., 2011). Beneficial effects of inclusion of homologous SP in freezing extenders on subsequent pregnancy rates were also reported for mares (Alghamdi et al., 2004) and sows (Rozeboom et al., 2000). However, as the use of ram SP is limited by its low volume in ejaculates, heterologous SP from bulls (Ollero et al., 1997) and stallions (Martins et al., 2013) have been tested as additives in freezing extenders for ram sperm.

Considering the great volume of boar SP in ejaculates and the potential beneficial effects of the protein content in SP for boar sperm quality after thawing (Rozeboom et al., 2000; Corcini et al., 2012), we hypothesize that boar SP could be an effective heterologous additive for extenders to freeze sperm from other species. Despite of some homology (nearly 22.0%) among SP proteins present in ejaculates of rams and boars (Druart et al., 2013), inclusion of boar SP in freezing extenders for ram sperm has not yet been tested. The objective of this study was to evaluate the effect of the inclusion of boar SP in freezing extenders on the quality of ram sperm after thawing.

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2. Material and methods

All chemicals used in this study were obtained from Sigma Chemical Company (St. Louis, MO, USA) except when mentioned differently.

2.1. Processing of seminal plasma

Four adult crossbred rams (in average six years old) were used in this study. The rams were kept under semi-extensive conditions at Embrapa Clima Temperado, in Pelotas-RS, Brazil. (31°45′S latitude and 52°21′W longitude), receiving concentrated diet twice daily and with *ad libitum* access to water. Four ejaculates were collected using an artificial vagina (one per ram), in July and August (Winter in the Southern hemisphere) and kept at 37 °C. The SP was extracted only from ejaculates with sperm motility equal or greater than 70%. Ejaculates were centrifuged twice at 12,500*g* for 10 min, in a refrigerated centrifuge at 4 °C. From each ejaculate, 400 μL aliquots of SP were collected, pooled and frozen at −20 °C.

Boar SP was obtained from ejaculates collected from three sexually mature crossbred boars, in average 16 months-old, through the gloved-hand technique (one per boar). The boars were housed at the Universidade Federal de Pelotas, in individual pens, receiving a 14% crude protein diet twice daily and water *ad libitum*. The SP was collected only from the sperm-rich fraction, from ejaculates with at least 70% sperm motility. Each ejaculate was split in fifteen aliquots (1.0 mL each), centrifuged at 2500*g* for 5 min. Thereafter, the supernatant was centrifuged at 10,000*g* for 10 min. Both centrifugations were done under refrigeration (4 °C). The SP samples were pooled and frozen at −20 °C.

The total protein concentration in SP pools from both species was determined through spectrophotometry (Bradford, 1976). Samples were evaluated in triplicate, in ultrapure water at 1:100. As protein concentration in SP was nearly four times greater for rams (188 μg/mL) than for boars (48 μg/mL), ram SP was diluted in Tris to adjust its protein content to 48 μg/mL.

2.2. Processing and evaluation of ram sperm

Ejaculates were collected from the same rams used for collection of SP (five ejaculates per ram), as mentioned above, during October (Spring in the Southern hemisphere). Prior to freezing, motility was evaluated through optical microscopy and spermatozoa concentration was determined with a Neubauer chamber. Ejaculates were extended to a concentration of 200×10^6 spermatozoa/mL in Tris-egg yolk glycerol (Evans and Maxwell, 1987) and fractioned in three treatments supplemented with: no SP (control); ram SP; and boar SP (both at 20% of the extender's total volume, containing 48 μg protein/mL). Sperm samples were stored in 0.25 mL straws and cooled at 0.3–0.5 °C/min until 5 °C, in a portable cooler (Igloo® Kool Mate 40, S. King Company Inc., Kenosha, Wisconsin, USA). After stabilization for 90 min at 5 °C, sperm samples were kept in N vapor for 10 min and then submerged in liquid N. For evaluation, straws were thawed in water bath at 37 °C for 30 s.

All sperm quality evaluations were conducted by the same trained technician. Sperm motion parameters were evaluated using a computer assisted semen analyses system (SpermVision®, Minitube, Tiefenbach, Germany) in an optical microscope (Axio Scope A1®, Zeiss, Jena, Germany) at 200×. Spermatozoa were evaluated in six automated randomized fields, considering: total and progressive sperm motility; distance average path (DAP); distance in a curved line (DCL); distance in a straight line (DSL); velocity average path (VAP); velocity in a curved line (VCL); velocity in a straight line (VSL); straightness (STR); linearity (LIN); wobble (WOB); amplitude of lateral head displacement (ALH); and beat cross frequency (BCF).

Sperm membrane integrity, acrosomal status, mitochondrial membrane potential and apoptotic-like changes were evaluated through flow cytometry (Attune®, Life Technologies, Eugene, OR, USA), considering an in average 10,000 spermatozoa in each evaluation. For such analyses, a volume of thawed sperm containing approximately 2×10^6 spermatozoa was added to 300 μL of a PBS-based solution containing specific fluorophores, as described below.

Sperm membrane integrity and acrosome status were evaluated as described elsewhere (Martinez-Pastor et al., 2005). Samples were incubated at room temperature for 15 min in a solution containing: 0.001 mg/mL of propidium iodide (PI) for evaluation of membrane integrity; 0.001 mg/mL of *Arachis hypogaea* FITC-PNA conjugate for evaluation of acrosome status; and 0.0025 mg/mL of the Hoechst 33342 solution, to separate sperm populations from debris. Samples were evaluated under blue laser (wave length of 488 nm), simultaneously at the BL3 filter (640LP) for PI and at the BL1 filter (530/30) for FITC-PNA, and under violet laser (wave length of 405 nm) at the VL-1 filter (450/40) for Hoechst 33342 (Alcaide et al., 2009). Spermatozoa with damaged sperm membrane cannot exclude IP, which binds to sperm's DNA producing red fluorescence. Spermatozoa with intact acrosome present green fluorescence due to staining with FITC, while PNA binds only to sperm cells with damaged acrosome. Simultaneous red and green fluorescence indicates damage in both membrane and acrosome.

The mitochondrial membrane potential was evaluated using 0.0019 mg/mL of the JC-1 dye (Life Technologies, Eugene, OR, USA), which produces fluorescence from green to orange (Martinez-Pastor et al., 2004). Samples were incubated at 37 °C for 30 min. Evaluations were done with violet laser (wave length of 405 nm) at the VL-3 filter (603/48) (Perelman et al., 2012). Spermatozoa with high mitochondrial membrane potential present high orange fluorescence and those with low potential present high green fluorescence. Intermediate potential considers cells with simultaneous orange and green fluorescence.

Apoptotic-like changes were evaluated as described by García-Álvarez et al. (2009). Samples were incubated for 15 min at room temperature in a solution containing 0.2 μL/mL of the YO-PRO-1 nuclear marker (Life Technologies, Eugene, OR, USA), 0.001 mg/mL of PI and 0.0025 mg/mL of Hoechst 33342. Evaluations were done with blue laser (488 nm) concurrently for PI at the BL3 filter (640LP) and for YO-PRO-1 at the BL1 filter (530/30). For Hoechst 33342, evaluation was done with violet laser (405 nm) at the VL-1 filter (450/40) (Alcaide et al., 2009). The IP stains necrotic spermatozoa with damaged membrane producing red fluorescence but it does not stain spermatozoa with increased membrane permeability, which is an indicator of apoptosis. The green fluorescence present in the nucleus of apoptotic cells is due to staining with YO-PRO, while non-apoptotic spermatozoa remain unstained.

2.3. Statistical analyses

As the Shapiro-Wilk test indicated lack of normality, all responses were submitted to arcsine transformation. Subsequently, transformed data were compared among treatments using analyses of variance with repeated measures, with the effect of individual rams nested within the effect of sperm collections. Comparisons of means were done with the Tukey test. To allow interpretation, results were reported in their original scales. All analyses were done with Statistix® (2013).

2.4. Results

When treated with either ram or boar SP before freezing, the ram sperm showed decreased post-thawing motion parameters ($P < 0.05$), such as the total and progressive motility, compared with