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Boar seminal plasma inhibits cryo-capacitation of frozen-thawed ram sperm and improves fertility following intracervical insemination

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ABSTRACT

Cryopreservation has numerous deleterious effects on sperm structure and function, which can be reduced by adding seminal plasma (SP), either autologous or heterologous. The objective was to determine effects of adding boar SP to the freezing extender on sperm quality, tyrosine phosphorylation and fertilizing ability of frozen-thawed ram sperm. Semen was collected from eight Small-tail Han rams and extended in a glucose-egg yolk buffer supplemented with 0, 20, 40, or 60% porcine SP (from Large white boars). Compared to all other groups, 40% boar SP increased sperm viability and motility ($P < 0.05$), whereas 20% boar SP had no beneficial effect, and 60% SP reduced sperm quality and motility ($P < 0.05$). Compared to control (0%), 40% boar SP inhibited cryo-capacitation and tyrosine phosphorylation of frozen-thawed ram sperm, and improved the proportion of capacitated sperm and tyrosine phosphorylation after *in vitro* capacitation ($P < 0.05$). Furthermore, based on viscous medium penetration tests, 40% boar SP increased sperm penetration (944.7 ± 121.5 *vs* 555.3 ± 88.7; $P < 0.05$). Finally, 40% boar SP improved pregnancy rate for intracervical AI (47.5 *vs* 33.3%; $P < 0.05$), apparently due to inhibition of cryo-capacitation, although pregnancy rate for intravaginal AI was not affected (31.6 *vs* 30.0%).

1. Introduction

The success of artificial insemination (AI) with frozen-thawed semen not only depends on fertilizing capacity, but also on sperm transport and survival in the female reproductive tract [1]. However, ovine sperm are extremely sensitive to cryopreservation, which causes structural and biochemical damage [2], thereby decreasing motility, morphological integrity, and mitochondrial membrane potential, and increasing chromatin damage and production of reactive oxygen species [3]. Freezing and thawing procedures also cause premature induction of a capacitation-like status in ram sperm, including rising tyrosine phosphorylation and intracellular calcium concentrations [4].

Various cryoprotectants have been studied to improve the quality and fertility of frozen-thawed ovine semen [5–7]. Seminal plasma (SP), a mixture of fluid secreted in the testis, epididymis, and accessory sex glands, contains several factors that modulate sperm function [8]. It has been reported that supplementation of frozen-thawed ram sperm with autologous (same species) SP improved sperm characteristics, including motility, viability [9–11], the ability to penetrate cervical mucus [12], and fertility after intrauterine insemination [13]. However, ram semen contains relatively little SP, which makes it impractical for large-scale use. Therefore, there is a need for functional alternatives. It is noteworthy that adding rainbow trout SP to ram semen [14], bull SP to African buffalo semen [15], ram SP to goat semen [16], and boar SP to bull semen [17], improved quality and fertility of frozen-thawed sperm, providing evidence that SP from one species can have cryoprotectant effects in another species. Based on large volumes of boar SP in each ejaculate and potential beneficial effects to improve frozen-thawed sperm quality in various species [17,18], we hypothesized that boar SP improves quality of frozen-thawed ram sperm. Therefore, the objective was to determine the effects of boar SP on quality, capacitation and fertilizing ability of frozen-thawed ram sperm.

2. Materials and methods

The use of animals and all experimental procedures were

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approved by the Animal Care Committee of the Institute of Geography and Agroecology, Chinese Academy of Sciences, Jilin, China. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.1. Rams and diet

Eight Small-tail Han sheep rams (age, 3.0–4.0 y; body weight, 75–80 kg) were used. All rams had *ad libitum* access to water and a ration of 60:40 forage: concentrate (as recommended by the National Research Council).

2.2. Sperm

2.2.1. Semen collection

Ejaculates were collected twice weekly for 5 wk, with 40 s ejaculates (the first ejaculates were discarded) obtained from the eight rams. Ejaculates with volumes of 0.7–2.0 mL, sperm concentrations >2.5 × 10^9 sperm/mL (/mL) and >80% motility were retained and pooled.

2.2.2. Collection of seminal plasma

Semen was collected from four Large white boars (2.0–3.0 y old) and centrifuged at 3000 × *g* for 30 min. The sperm-free supernatant was removed, filtered through a membrane (Pall Corporation, New York, NY, USA) with 0.22 μm pores, and stored at −80 °C.

2.2.3. Semen dilution, freezing, and thawing

The basic semen extender (Tris-egg-yolk) contained an 80% (v:v) solution of 3.0% glucose, 3.0% sodium citrate, penicillin (10 IU/mL), and streptomycin (10 IU/mL) in distilled water and 20% (v:v) of egg yolk (fresh). This extender was supplemented with 0 (Control), 20, 40 or 60% (v:v) of boar SP. The freezing solution contained 94% (v:v) of extender and 6% (v:v) of glycerol.

All ejaculates were mixed thoroughly and incubated at 37 °C in a water bath for 30 min. The pool was divided into four aliquots, which were diluted to 1.0 × 10^9/mL with various concentrations of boar SP freezing solutions, then placed in 0.25 mL straws, and chilled at 4 °C for 2 h. Then, straws were placed 3–4 cm above liquid nitrogen for 7 min and subsequently plunged into liquid nitrogen. Samples were stored for at least 2 wk and subsequently thawed (40 °C water bath for 15 s) to perform the subsequent analyses.

2.3. Assessment of sperm quality

The concentration of frozen-thawed semen was determined with a sperm density meter (SDM1, Minitube, Germany) after dilution to 1 × 10^6/mL with phosphate-buffered saline (PBS), and kept in a water bath at 37 °C for all evaluations. Assessments of sperm vitality, acrosome integrity, plasma membrane integrity, mitochondrial activity and capacitation were conducted by the same trained technician and were repeated five times.

2.3.1. Vitality

Sperm vitality was assessed with the eosin-nigrosin stain method. The stain was prepared by dissolving 1.40% eosin-Y (Beyotime, Jiangsu, China), 8.72% nigrosine (Beyotime), and 2.53% sodium citrate in distilled water. Smears were prepared by mixing 30 μL of sperm suspension with 100 μL of eosin-nigrosin stain on a warm slide and spreading the stain with a second slide, followed by co-incubation at 37 °C for 2 min. Five non-consecutive microscopic fields were randomly chosen on the slide and a total of 200 cells per slide and three slides per sample were examined under 400 x magnification using a phase contrast microscope (Olympus BX60, Olympus, Tokyo, Japan). Sperm with partial or complete purple staining were considered nonviable, whereas only sperm with complete exclusion of stain were considered viable.

2.3.2. Acrosome integrity

A 100-μL aliquot of frozen-thawed sperm suspension was fixed in 500 μL 1% formal citrate (2.79% trisodium citrate dehydrate and 0.37% formaldehyde in 100 mL distilled water) at 37 °C for 20 min. Then, a 100-μL drop of the mixture was spread on a warm slide with a coverslip. Five non-consecutive microscopic fields were randomly chosen on the slide and a total of 200 cells per slide and three slides per sample were examined under 1000 x magnification using a phase-contrast microscope (Olympus BX60). A normal acrosome was characterized as having a normal apical ridge. The following sperm abnormalities were characterized: head defects, including micro- and macro-heads, detached heads, pyriform heads; mid piece abnormalities including proximal droplet, distal droplet and abaxial attachment; tail abnormalities like tail coiled below the head, tail bent at mid piece, tail without head and double tail [19].

2.3.3. Plasma membrane integrity

The hypo-osmotic swelling test was used to determine functional integrity of the sperm membrane, based on curled and swollen tails. In brief, 50 μL of frozen-thawed sperm suspension was incubated with 300 μL of hypo-osmotic solution (76.6 mM fructose and 28.5 mM sodium citrate in distilled water) at 37 °C for 60 min. Then, a 100-μL drop of the mixture was spread on a warm slide with a coverslip. Five non-consecutive microscopic fields were randomly chosen in the slide and a total of 200 cells per slide and three slides per sample were examined under 1000 x magnification using a phase contrast microscope (Olympus BX60). Sperm with tail curling (%) were considered as having an intact plasma membrane [20].

2.3.4. Mitochondrial activity

To assess mitochondrial activity, 5 μL of 2.0 mM JC-1 (Invitrogen, Camarillo, CA, USA) staining solution was added to 1 mL of frozen-thawed sperm suspension (JC-1 final concentration of 9.95 μM). Sperm were incubated at 37 °C water bath for 30 min in the dark. Then, 5 μL of 1.0 mM PI (Invitrogen, Camarillo, CA, USA) staining solution was added to diluted samples and they were incubated for 5 min in the dark. Thereafter, samples were centrifuged at1000 × *g* for 10 min, the supernatant discarded, and PBS added to adjust sperm concentration to 1 × 10^6/mL using sperm density meter (SDM1, Minitube). Mitochondrial activity was determined with flow cytometry (Becton Dickinson, Sunnyvale, CA, USA). Data for 10,000 sperm per sample were stored in the list mode using FACS Analyzer flow cytometry software (Becton Dickinson); thereafter, these data were passed through a Hewlett Packard (Palo Alto, CA, USA) Consort 30 and analyzed by SuperCyt Analyst 3 software (Sierra Cytometry, Reno, NV, USA).

2.4. Analysis of sperm kinematics using CASA

For each sample of frozen-thawed semen, a computer-aided sperm analysis system (CASA, Minitube) was used to determine: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), progressive velocity (VSL, μm/s), and path velocity (VAP, μm/s). In brief, sperm concentration of frozen-thawed semen was calculated using a sperm density meter (SDM1, Minitube) after dilution to 2.0 × 10^7/mL in PBS in each group and incubation in a water bath at 37 °C for 5 min. Then, a 5-μL drop of sample was placed on a preheated glass slide (37 °C). For each sample, five non-consecutive microscopic fields were randomly chosen on the slide and three slides per sample were examined