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Seminal plasma applied post-thawing affects boar sperm physiology: A flow cytometry study

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ABSTRACT

Cryopreservation induces extensive biophysical and biochemical changes in the sperm. In the present study, we used flow cytometry to assess the capacitation-like status of frozen-thawed boar spermatozoa and its relationship with intracellular calcium, assessment of membrane fluidity, modification of thiol groups in plasma membrane proteins, reactive oxygen species (ROS) levels, viability, acrosomal status, and mitochondrial activity. This experiment was performed to verify the effect of adding seminal plasma on post-thaw sperm functions. To determine these effects after cryopreservation, frozen-thawed semen from seven boars was examined after supplementation with different concentrations of pooled seminal plasma (0%, 10%, and 50%) at various times of incubation from 0 to 4 hours. Incubation caused a decrease in membrane integrity and an increase in acrosomal damage, with small changes in other parameters ($P > 0.05$). Although 10% seminal plasma showed few differences with 0% (ROS increase at 4 hours, $P < 0.05$), 50% seminal plasma caused important changes. Membrane fluidity increased considerably from the beginning of the experiment, and ROS and free thiols in the cell surface increased by 2 hours of incubation. By the end of the experiment, viability decreased and acrosomal damage increased in the 50% seminal plasma samples. The addition of 50% of seminal plasma seems to modify the physiology of thawed boar spermatozoa, possibly through membrane changes and ROS increase. Although some effects were detrimental, the stimulatory effect of 50% seminal plasma could favor the performance of post-thawed boar semen, as showed in the field (García JC, Domínguez JC, Peña FJ, Alegre B, Gonzalez R, Castro MJ, Habing GG, Kirkwood RN. Thawing boar semen in the presence of seminal plasma: effects on sperm quality and fertility. *Anim Reprod Sci* 2010;119:160–5).

1. Introduction

The achievement of successful semen cryopreservation would represent a dramatic leap in swine production systems. This would allow to the widespread use of germplasm banks for storing semen doses from selected males, for preserving genetic diversity, and for conserving rare breeds. Moreover, the long-term availability of frozen semen doses would allow for more flexible and efficient breeding programs, and it would help to control the transmission of pathogens [1]. Despite the utilization of refrigerated long-term semen storage, the use of cryopreserved boar semen has not achieved widespread acceptability for commercial breeding by artificial insemination, mainly for economical and political reasons, although there is need for technical improvement too [2]. It is possible to achieve fertility results comparable to fresh semen by combining cryopreservation with deep intrauterine insemination and accurate estimation of ovulation [3]. However, the use of thawed semen results in decreased farrowing rates and litter size if the insemination occurs outside this ovulation time window. Environmental and management factors affect fertility of thawed semen more than when using refrigerated semen [4,5].

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The damage to boar sperm caused by cryopreservation includes motility impairment, chromatin damage, membrane alterations, and decreased mitochondrial membrane potential, caused by cold shock, osmotic shock, and oxidative damage by reactive oxygen species (ROS) to which boar spermatozoa seem to be especially sensitive [6,7]. Several factors have been presented as the cause of the low or irregular fertility results of frozen-thawed boar spermatozoa, including premature capacitation-like changes during the process of cooling and cryopreservation [6,8]. These changes have been termed as "cryocapacitation," and could shorten the life span of spermatozoa, modify regulation pathways, cause early acrosome reaction, and modify the plasma membrane, resulting in part of the sperm population being unable to interact with the oviduct or to fertilize the ovum [9].

Using seminal plasma could help to improve semen quality after thawing. It is known that seminal plasma affects the physiology of spermatozoa, although its effects on different species are very variable, depending also on the condition of the sample [10–13]. In the case of boar spermatozoa, incubation of fresh or cryopreserved sperm in media supplemented with 10% seminal plasma seems to prevent, and possibly reverse, capacitation-related changes [7,8,14]. In several studies, the post-thawing addition of seminal plasma improved membrane and acrosomal integrity, and enhanced the *in vivo* fertilization [15]. Use of 10% seminal plasma after thawing rendered good fertility results when combined with a modified freezing/thawing protocol [16].

However Abad et al. [17] found that 10% seminal plasma supplementation did not affect the creation of the oviductal sperm reservoir. The same authors reported no improvement on sow fertility when thawed boar semen was supplemented with 10% seminal plasma and inseminated by 2 or 12 hours of the predicted time of ovulation [18]. Therefore, they concluded that seminal plasma could not completely reverse cryocapacitation, or else other factors were having a role, decreasing cryopreserved semen fertility.

The objective of the present study was to enhance our understanding on the effects of post-thawing addition of seminal plasma to boar semen by analyzing several physiological variables using flow cytometry. This study follows a previous trial in which adding 50% seminal plasma to thawed boar semen made both pregnancy rate and mean litter size comparable to those achieved with liquid-stored semen [19]. Taking this study as a starting point, we posed the hypothesis that the seminal plasma would modify the physiology of thawed spermatozoa, explaining the fertility improvement found by [19]. Thus, we assessed the spermatozoa during a 4-hour incubation in the presence of 50% seminal plasma. A concentration of 10% seminal plasma was included in the study because it has been used in most studies, although it has yielded mixed results.

2. Materials and methods

2.1. Experimental design

The spermatozoa used in this experiment were obtained from frozen semen doses stored in our cryobank. After thawing, the pooled contents of two straws were diluted down to 25×10^6/mL with MR-A extender (Kubus S.A., Madrid, Spain), split among three 1.5-mL tubes, and supplemented with 0%, 10%, or 50% of heterologous seminal plasma. The tubes were incubated at 37 °C and assessed each hour (sampling points at 0, 1, 2, 3, and 4 hours). At each sampling point, an aliquot from each tube was mixed with fluorescence probes for assessing several physiological parameters: stability of the plasma membrane (damage, apoptotic-like changes, and fluidity), acrosomal damage, mitochondrial activity, intracellular Ca^{2+} concentration, intracellular ROS concentration, and abundance of extracellular free thiols.

2.2. Reagents and media

Fluo-4 AM cell permeant, merocyanine 540 (M540), propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), 5-iodoacetamidofluorescein (5-IAF) Mitotracker Deep Red, and YO-PRO-1 iodide used in the study were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals such as fluorescein isothiocyanate-peanut agglutinin (FITC-PNA), DMSO, and PBS were purchased from Sigma (St. Louis, MO, USA). Stock solutions of fluorescence probes were prepared in DMSO at 1 mM, except YO-PRO-1 (25 μM), PI (1 mg/mL in water), and PNA-FITC (0.2 mg/mL in water). These stocks were kept at −20 °C in the dark. Flow cytometry equipment, software, and consumables were purchased from Becton Dickinson (San Jose, CA, USA).

2.3. Semen collection and preservation

Semen was collected from seven mature Landrace, Large White, and Duroc boars by the "gloved-hand method." For each ejaculate, the sperm concentration was determined using a spectrophotometer. The initial percentage of motile sperm was determined visually and any ejaculates containing >60% motile sperm were used.

Semen was processed for cryopreservation according to the technique described by Eriksson and Rodríguez-Martínez [20], except that our first extender dilution was MR-A at 2:1 rather than Beltsville Thawing Solution at 1:1, and we used slower cooling and freezing curves [21]. Initially, 60×10^9 sperm from each ejaculate were diluted in MR-A previously warmed to 32.5 °C. The extended semen was incubated at room temperature (20 °C–22 °C) for 1 hour. Then, the semen was transferred to a room at 15 °C for 3 hours, centrifuged at $800 \times g$ for 10 minutes at 15 °C in a programmable refrigerated centrifuge (Heraeus Megafuge 1.0 R, Heraeus Holding GmbH, Germany), and the supernatant was discarded. The pellets were reextended with lactose-egg yolk extender (80% (vol/vol) of a 11.0% (wt/vol) lactose monohydrate solution, and 20.0% (vol/vol) of hen's egg yolk) to a final concentration of 1.5×10^9 spermatozoa/mL. After thorough mixing, the semen was cooled for 2 hours in a refrigerator at 5 °C. At this temperature, the semen was slowly mixed with the freezing extender, consisting of 89.55% (vol/vol) lactose-egg yolk extender, 8.95% (vol/vol) glycerol, and 1.5% (vol/vol) Equex STM (Minitüb, Germany) at a ratio of two parts of semen to one part of extender, yielding a final concentration of 3% glycerol and 1×10^9 spermatozoa/mL.

Sperm were packaged at 5 °C in 0.25 mL straws. After sealing, all racks were transferred to the chamber of