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The protective effect of a 17 °C holding time on boar sperm plasma membrane fluidity after exposure to 5 °C

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ABSTRACT

The holding time (HT) is the period during which an ejaculate, either in a raw or diluted state, is held at 17 °C before further processing for cold-storage. In boars, the HT positively influences select sperm quality parameters of semen cooled from 15 to 5 °C, a range in temperature during which plasma membrane remodeling occurs. Objective insight into the effect of HT on plasma membrane organization remains unknown. Therefore, the present work sought to elucidate if HT contributes to minimizing alterations in boar sperm plasma membrane fluidity at the initial step of the cooling process in a cryopreservation practice (holding at 5 °C) and in relation with select sperm quality parameters. Nineteen ejaculates from five boars were collected and processed according to different treatments: T1) Fresh diluted semen, 0 h at 17 °C; T2) Fresh diluted semen, 24 h at 17 °C (HT); T3) Sperm from T1 in a lactose-egg yolk (LEY) extender, 3 h at 5 °C; T4) Sperm from T2 in LEY, 3 h at 5 °C; T5) Sperm from T1 in LEY, 24 h at 5 °C; T6) Sperm from T2 in LEY, 24 h at 5 °C. Sperm motility was assessed using CASA, and sperm plasma membrane integrity and fluidity were evaluated by flow cytometry with dual labeling (M540/YO-PRO®-1). Results demonstrated that the lack of exposure to a HT (T5) results in reduced sample motility compared to those having a HT (T6), with sperm exposed to HT exhibiting less plasma membrane fluidity. Collectively, these results provide empirical evidence that incorporation of a HT in semen processing protects boar sperm against cold injury through maintenance of lipid architecture of the plasma membrane.

Introduction

Pursel, Schulman and Johnson [34] initially described the benefits of incubating freshly collected gel-free boar ejaculates at room temperature (15–30 °C) prior to further extension in a diluent and followed by cooling and long-term storage. Specifically, they found that ejaculates held for six hours at room temperature demonstrated improved sperm motility and acrosome integrity over those ejaculates which were collected and immediately processed to 5 °C.

This observation is of particular interest in the pig industry for the purpose of cryopreservation and also in view of the new extender formulations that are being sought to hold pig semen at 5 °C. This storage temperature would slow down the sperm metabolism and thus preserve their functionality for longer than if stored at 17 °C, allowing as well better control of bacterial contamination. Whereas other mammalian sperm are already being stored at 5 °C [4,43] pig sperm is still being held at 15–17 °C given the sensitivity a percentage of ejaculates shows when cooled to this point [2,10,24].

Implementation of a holding time (HT) has thus become a component of boar semen cryopreservation protocols and it could be regarded as well as a tool to help introduce in practice the storage at the 5 °C. Certainly, the standard cryopreservation practice has adopted techniques to reduce the extent of cooling damage, like inclusion of egg yolk in the extender and adjustment of the cooling rate, but the necessity of incorporating a HT in the processing of boar semen provides evidence that cold injuries still occur when lowering the sample temperature from 15 to 5 °C. Today, a minimal HT of 1–3 h in conventional extender before cryopreservation is recommended for boar sperm [6,42], although most protocols include variations up to a 24-h HT to accommodate for such things as overnight courier to the processing laboratory [19,20].

Elucidation into the mechanisms by which the HT may contribute to a more successful outcome after cooling remains largely unsolved. In pig sperm, different domains in the sperm membrane can present variable responses to chilling temperatures, but it is assumed that a general increase in fluidity occurs after the cryopreservation process [5,31–33]. This increase is believed to be due to the effect that temperatures of 5 °C and below have on membranes, particularly on the formation of lipid clusters that break down the bilayer selective permeability [44]. It is suggested that the HT somehow minimizes sensitivity to chilling when applied to either extender-diluted or raw ejaculates [6,4], possibly through contributory effects of select

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seminal plasma proteins [25]. As such, dilutions under ten-fold with extender are common practice before freezing to facilitate the interaction of seminal plasma and its components with the sperm's plasma membrane [34,16,19,20].

The lipidic arrangement on the plasma membrane during HT exposure is proposed to be a major factor in explaining the higher tolerance of boar sperm to cold injuries [44,16,18,41]. Because the distribution of lipids in the bilayer, or membrane fluidity, modulates vital metabolic functions within the cell, the plasma membrane fluidity is frequently used as an early indicator of cellular damage [26,45,25]. It is known that the boar sperm plasma membrane undergoes remodeling during cooling to 5 °C or below, which diminishes the lifespan of the cell compared to samples held at 15 °C [15,14,44,19,20]. To our knowledge no empirical evidence has been provided to date to fully explain the actual effects of HT on boar sperm plasma membrane fluidity during cooling.

Therefore, the objective of the present work was to determine if application of a defined HT on diluted boar sperm modulates lipid reordering within the first step of a cryopreservation procedure (17–5 °C), as measured via plasma membrane fluidity, and to evaluate the relation of the defined HT on select sperm quality parameters after cold exposure (5 °C) and its future implications on the storage of sperm at 5 °C.

Material and methods

Experimental setup

The experiment was designed to compare the effects of the HT on the sperm quality and plasma membrane fluidity between three different paired treatments related to the first cooling steps of a boar sperm cryopreservation process: T1 vs T2, T3 vs T4, and T5 vs T6. These treatments were the following: (T1) Fresh diluted semen, 0 h at 17 °C; (T2) Fresh diluted semen, 24 h at 17 °C (HT); (T3) Sperm from T1 in lactose-egg yolk (LEY) extender, 3 h at 5 °C; (T4) Sperm from T2 in LEY, 3 h at 5 °C; (T5) Sperm from T1 in LEY, 24 h at 5 °C; (T6) Sperm from T2 in LEY, 24 h at 5 °C.

By this way, a schedule was established so that treatments without a HT, and therefore lacking incubation with seminal plasma, were analyzed within the collection day (day 0; T1 and T3) and on day 1 (T5), while treatments with a HT and, thus, exposed to seminal plasma, were assessed 24 h later (T2 and T4 were assessed on day 1, and T6 on day 2).

Treatments T1 and T2 sought to evaluate if a HT affected the quality and plasma membrane fluidity of sperm early after collection and before further handling. Treatments T3 and T4, which incorporated a cooling rate from 15 to 5 °C as used in conventional cryopreservation procedures [9], were applied to confirm if the HT accounted for differences in the sperm quality and plasma membrane fluidity after this cooling step in LEY extender. Treatments T5 and T6 sought to check if the HT provided differences in the sperm quality and plasma membrane fluidity after sperm had been challenged to long-term storage conditions at 5 °C in LEY extender.

Semen collection

Five mature crossbred boars (large white x landrace x duroc) ranging in age 10–16 months were used in the study. All boars were housed individually under controlled conditions in a 2.5 × 1.5 m space. Animals were fed a standard diet (21% CP) with *ad libitum* access to water. During the experimental period, boars were on a once per week semen collection schedule. Nineteen gel-free ejaculates were collected into an insulated thermos using the gloved-hand technique while boars were mounted on a dummy sow [1].

After initial estimation of sperm concentration using a calibrated photometer [1], ejaculates were diluted 1:7 (v:v) in a conventional extender and transported within 60 min post-collection to the laboratory inside an insulated foam box held at ambient temperature (15–17 °C). At the laboratory, each diluted ejaculate was sub-divided into two aliquots corresponding to treatments T1 and T2. The aliquot destined to the HT treatment (T2) was held for 24 h at 17 °C, whereas the other aliquot (T1) was immediately assessed. Samples for treatments T3–T6 were taken from these aliquots as specified in the experimental setup section.

Briefly, samples for T3–T6 consisted of 50 mL aliquots that were centrifuged at 640*g* for 3 min to remove the previous extender. The supernatant was discarded and the soft pellet re-suspended in LEY freezing medium to achieve a final concentration of 1.5×10^9 -sperm mL^{-1}. Sperm was cooled over a three hour period to 5 °C inside closed tubes submerged in isothermal water and placed in a standard refrigerator (cooling rate averaging 0.06 °C min^{-1}). Sperm quality was checked after this time period for the evaluation of treatments T3 and T4, and after 24 h at the 5 °C for the assessment of treatments T5 and T6.

Assessment of sperm motility

Sperm motility was assessed using an Integrated Visual Optical System (IVOS v12.3D, Hamilton Thorne Inc., Beverly, MA, USA). Before warming to 37 °C samples from aliquots corresponding to treatments T1 and T2 were diluted 1:1 (v:v) in a commercial extender (Gedil®, Genes Diffusion-IMV Technologies, Douai, France). When analysis was performed on samples from the remaining treatment groups (T3–T6; sperm cooled in LEY), those were diluted 1:19 (v:v) in the commercial extender to clean the visual field from egg yolk particles. At least 1000 spermatozoa were counted per analysis. The sperm kinematic parameters that were collected are described in Table 1.

Assessment of sperm integrity and plasma membrane fluidity

The percentage of intact sperm in terms of plasma membrane damaged and apoptotic cells and the plasma membrane fluidity were screened following previously validated protocols [21,41] using a FACScan™ flow cytometer with CellQuest™ Pro software (BD Biosciences, San Diego, CA, USA). Samples were diluted to 5×10^6 sperm cells mL^{-1} in extender and 1 mL of the dilution was incubated at 37.5 °C and 5% CO_2 for 10 min with 1 µl YO-PRO®-1 iodide (25 µM) and 2.6 µl merocyanine 540 (M540, 1 mM) (Molecular Probes, Invitrogen, Eugene, OR, USA). The M540 is a lipophilic probe that binds preferentially polarized membranes whose lipids are more widely spaced, thus emitting intense fluorescence upon excitation as plasma membrane fluidity increases [21,45]. The YO-PRO®-1 iodide is a nuclear dye that permeates only apoptotic and/or plasma membrane damaged cells.

After experimental setting of the acquisition parameters using positive and negative fluorescence and background controls, the

Table 1
Description of the kinematic parameters included in the sperm motility assessment. System settings were established to capture 45 video frames at 60 frames per second and a minimum cell size of 7 pixels.

Total motility	Motile sperm cells in the sample	%
VAP	Average path velocity	µm s^{-1}
VSL	Straightlinear velocity	µm s^{-1}
VCL	Curvilinear velocity	µm s^{-1}
ALH	Amplitude of lateral head displacement	µm
BCF	Beating frequency	Hz
STR	Straightness (VSL $VAP^{-1} \times 100$)	%
LIN	Linearity (VSL $VCL^{-1} \times 100$)	%
Progressive motility	Sperm cells showing ⩾ 45% VAP and STR	%