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Boar variability in sperm cryo-tolerance after cooling of semen in different long-term extenders at various temperatures

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ABSTRACT

This study investigated individual boar variability in the quality of pre-freeze (PF) and post-thaw (PT) semen cooled in different long-term (LT) extenders and for different holding times (HT). Sperm rich fractions were diluted with Androhep® Plus (AHP), Androstar® Plus (ASP), Safecell® Plus (SCP) and TRIXcell® Plus (TCP) extenders, stored for 2 h at 17 °C (HT 1) and additionally for 24 h at 10 °C (HT 2) and the samples were subsequently evaluated and frozen. Besides the analysis of CASA sperm variables, mitochondrial membrane potential (MMP), plasma membrane integrity (PMI), normal apical ridge (NAR) acrosome integrity, and viability (YO-PRO-1$^-$/PI$^-$) of sperm were assessed in the PF and PT semen. Results indicated that boar, extender and HT group affected the sperm quality characteristics. There were great variations in PMOT and the sperm motion patterns of the PF semen among the boars. Differences in the HT groups of the PF semen, with respect to the sperm membrane integrity, were less marked among the boars. Consistent variations in TMOT and PMOT in the PT semen were observed among the boars, being greater in the HT 2 group. Most of the CASA-analyzed sperm motion patterns were greater in the HT 2 group of the PT semen. Furthermore, sperm MMP, PMI and viability were greater in the HT 2 group of the PT semen in most of the boars, while consistent differences were observed among the boars for sperm NAR acrosome integrity in either HT group. The significant effect of the cryopreservation process on the sperm membrane proteome was evident from the number of protein bands, detected in the electrophoretic profiles of sperm of the HT 1 and HT 2 groups. The electrophoretic profiles of the PF and PT semen among boars with poor and good semen freezability, however, differed with respect to the abundance and types of sperm membrane-associated proteins. The overall results of this study provided evidence that there are differences among boars in response to the different cooling regimens, and that cooling of extended semen for a 24-h period at 10 °C modulated the functions of sperm in an extender-dependent manner, rendering the cells less susceptible to cryo-induced damage. It is suggested that the findings of this study have the potential to improve the technology of boar semen cryopreservation.

1. Introduction

Cryopreservation of semen has been considered as a method of storing the genetic potential of outstanding breeding animals for prolonged periods, and additionally it contributes to the conservation of endangered species (Holt, 2000). During cryopreservation, sperm are exposed to a stressful environment that causes irreversible damage to the structural and functional membrane integrity, resulting in a loss of viability and fertilizing capacity (Fraser et al., 2010; Yeste, 2015, 2016). Even with many advances in the

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cryopreservation of boar semen, the widespread use of frozen-thawed semen in the swine industry is still limited compared with liquid-stored semen (Knox, 2015; Yeste, 2015).

The cryopreservation protocol of boar semen consists of a multistep procedure, including extension and cooling periods, making the sperm vulnerable to the cold shock effect and semen processing, prior to freezing (Althouse et al., 1998; Holt, 2000; Yeste, 2016). The sperm cells in extended boar semen stored for a 60–72 h period at temperatures between 12 and 15 °C has a greater resistance to cold shock (Althouse et al., 1998; Chutia et al., 2014). Several studies have confirmed that a 24-h holding time (HT) period of extended boar semen at 17 °C increases sperm cryo-tolerance (Guthrie and Welch, 2005; Casas and Althouse, 2013; Alkmin et al., 2014, Yeste et al., 2014). Moreover, storage of boar semen in long-term (LT) extenders exerts beneficial effects on the quality of the pre-freeze (PF) and post-thaw (PT) semen (Kaeoket et al., 2011; Frydrychová et al., 2015). With this same approach, in a recent study where there was a 24-h HT period of diluted boar semen in different LT extenders at 10 °C, there was reduced sperm susceptibility to cryo-induced damage (Wasilewska et al., 2016). Even though studies indicate the cooling of boar semen in different LT extenders at the initial stage of the cryopreservation protocol improves the quality of PT semen (Kaeoket et al., 2011; Frydrychová et al., 2015), optimal sperm cryo-survival depends on the capacity of semen of individual animals to respond to the freezing-thawing conditions (Holt, 2000; Holt et al., 2005; Roca et al., 2006; Fraser et al., 2007, 2010). Most of the variation in boar sperm cryo-survival is due to male-to-male differences, suggesting that boar is a primary factor influencing semen freezability (Roca et al., 2006; Fraser et al., 2007; Alkmin et al., 2014; Yeste, 2016). The reason for the inherent boar variability in sperm cryo-survival is still not fully known, but it has been suggested that this is associated with molecular markers linked to genes controlling semen freezability (Thurston et al., 2002; Fraser et al., 2008). The present study was, therefore, undertaken to investigate the effects of different LT extenders and cooling regimens (2 and 24 h HT at 17 and 10 °C, respectively) on the quality of the PF and PT semen of individual boars. Also, the electrophoretic profiles of sperm membrane proteins were analyzed in the PF and PT semen of each boar.

2. Materials and methods

2.1. Chemicals and media

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. Androhep® Plus (AHP) and Androstar® Plus (ASP)were purchased from Minitübe (Tiefenbach, Germany), whereas Safecell® Plus (SCP) and TRIXcell® Plus (TCP) were purchased from IMV Technologies (L'Aigle, France).

2.2. Animal and semen collections

Sperm-rich fractions (SRFs) were collected from five Polish Landrace (average age 18 months), housed at the Cryopreservation Laboratory at the Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn (Poland). Five or seven SRFs were collected from each boar (designated as boars A, B, C, D and E), using the gloved-hand technique. The boars were fed the same commercial diet throughout the entire experimental period. Water was available *ad libitum*. Animal experiments were conducted in accordance with the guidelines of the Local Ethics Committee. The SRF of each boar had more than 70% total sperm motility (TMOT) and less than 15% of sperm cells had abnormal morphology. Sperm concentration was determined using a Bürker counting chamber (Equimed-Medical Instruments, Cracow, Poland).

2.3. Semen processing procedure

The SRFs were treated for processing similarly to the procedure reported in a previous study (Wasilewska et al., 2016). Briefly, the SRF from each boar was divided into four aliquots, diluted (100×10^6 sperm/ml) with the AHP, ASP, SCP and TCP extenders, and stored for 2 h at 17 °C (HT 1), prior to analysis and freezing. The remaining portion of the cooled semen was stored for 24 h at 10 °C (HT 2) and was subsequently evaluated and frozen. All cooled samples were processed, according to a previously described cryopreservation protocol using 5% lyophilized lipoprotein fractions of ostrich egg yolk, LPFo (Fraser et al., 2010). Following centrifugation ($800 \times g$ for 10 min) of the cooled semen (HT 1 and HT 2), the sperm pellets were re-suspended in the LPFo-extender containing 11% lactose (lactose-LPFo extender), was cooled to 5 °C for 2 h, and diluted (2:1) with a freezing extender containing 89.5 mL lactose-LPFo extender, 9 mL glycerol (v/v) and 1.5 mL Orvus Es Paste (OEP). The final sperm concentration was approximately 500×10^6 sperm/mL. Semen samples were packaged in 10-mL sterilized aluminum tubes, frozen in a controlled programmable freezer (Ice Cube 14 M, SY-LAB, Austria) and stored in liquid nitrogen, prior to thawing in a water bath for 60 s at 50 °C. Following thawing, the samples were diluted (1:10) with the AHP, ASP, SCP or TCP extender and incubated for 15 min at 37 °C prior to analysis.

2.4. Evaluation of sperm quality characteristics

2.4.1. Sperm motility and motion patterns analyzed by the CASA system

Aliquots of sperm samples (5 μL) were placed on a pre-warmed Makler counting chamber and examined at 37 °C, using the computer-assisted sperm analysis (CASA) system (HTR-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA). The sperm variables analyzed by the CASA system included total motility (TMOT, %), progressive motility (PMOT, %), velocity curvilinear (VCL, μm/s), velocity average pathway (VAP, μm/s), velocity straight line (VSL, μm/s), and rapid movement (RAP, %). A minimum of five fields