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Effect of the holding time at 15 °C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation

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ABSTRACT

The aim of the present study was to evaluate the effect of the holding time at 15 °C prior to cryopreservation (2, 4 and 8 h), thawing rate (37 °C for 20 s or 70 °C for 8 s) and post-thaw incubation temperature (15 °C or 37 °C) on the post-thaw boar sperm quality. These are important time periods in the freezing–thawing process which have been less studied. Sperm-rich ejaculate fractions from three healthy boars were collected once a week for five consecutive weeks and were cryopreserved with the lactose-egg yolk extender (LEY). Sperm quality was determined by assessing the motility, the acrosome status, and the sperm plasma membrane integrity at 30, 150 and 240 min of incubation. The results show that with the holding time at 15 °C prior to cryopreservation there was not a clear effect until at least 24 h of holding time. The thawing rate and the post-thaw incubation temperature, however, had a marked effect on sperm quality. When the samples were thawed at 70 °C for 8 s, the sperm viability, motility and some kinetic variables (VCL, VSL, VAP and ALH) were greater than with results observed when the samples were thawed at 37 °C for 20 s. In addition after thawing the sperm samples incubated at 15 °C had a sustained sperm quality for longer, up to 4 h post-thawing.

1. Introduction

Frozen-thawed boar semen is not widely used in the swine industry due to only approximately 50% of boar spermatozoa being adequately protected by current cryopreservation protocols (Watson, 1995). The results of insemination indicate a distinct reduction in farrowing rate and litter size when compared to use of fresh spermatozoa (Johnson et al., 2000).

This seminal quality loss is due to detrimental actions on cells during the cooling, freezing and thawing process of boar spermatozoa that result in extensive membrane and tail damage (Watson, 1981; Parks and Lynch, 1992). As a result, the spermatozoa that survive remain with sub-lethal dysfunctions (Watson, 2000) which reduce the longevity of sperm and fertilizing ability.

The damage to sperm is largely unavoidable because the physiology (composition of the membranes) of sperm that makes the cells particularly susceptible to the processing (Parks and Lynch, 1992; Watson, 1995). To address these problems, scientists have focused research on the most important processes such as the decrease of temperature from 15 °C where boar spermatozoa are particularly

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susceptible to cold shock (Watson, 1995). There has also been a focus on the freezing process with evaluation of different freezing rates (Eriksson and Rodriguez-Martinez, 2000; Holt et al., 2005) and the addition of agents that protect cells during cryopreservation (Gutiérrez-Pérez et al., 2009; Malo et al., 2012). However, other processes such as the holding time prior to the cryopreservation, thawing rate and post-thaw incubation temperature are also involved during freezing–thawing and have not been studied to a great extent.

Occasionally semen must be shipped to another location for cryopreservation. This semen is diluted in a conservation extender and is maintained at 15 °C for a maximum of 24 h prior to cryopreservation. This holding time prior to cryopreservation can have a beneficial effect because it has been observed that the incubation of sperm with the seminal plasma before freezing has a beneficial effect. This incubation improves resistance of sperm to cold shock or can result in a reversion of sperm capacitation processes (Aurich et al., 1996; Maxwell et al., 1999; Garner et al., 2001; Vadnais et al., 2005). Some studies, however, have indicated this incubation may reduce sperm function and fertility (Kawano et al., 2004; Moore et al., 2005; Akcay et al., 2006).

Another important point in the freezing–thawing process is the thawing rate (Mazur, 1965). Several studies have shown that a more rapid thawing rate results in greater sperm motility and acrosome integrity (Pursel and Johnson, 1975; Fiser et al., 1993; Eriksson and Rodriguez-Martinez, 2000; Córdova-Izquierdo et al., 2006; Muiño et al., 2008).

The other important processing aspect is the post-thaw temperature incubation. Usually the post-thaw incubation is at 37 °C, because this is the temperature at which artificial insemination is performed and the normal temperature in the female reproductive tract. This incubation temperature is normally used in the thermo-resistance test with 6–8 h of testing (Peláez et al., 2006; Gómez-Fernández et al., 2012; Carreira et al., 2012) because this is the average lifespan of frozen–thawed sperm when deposited in female genital tracts (Waberski et al., 1994). This technique is a valuable tool to analyze frozen-thawed sperm quality, but sometimes artificial insemination with frozen-thawed sperm doses is at a different location, and sperm are exposed to long incubations at 37 °C that can cause a progressive loss of sperm quality (Foley et al., 1967; Huo et al., 2002; Pérez-Llano et al., 2010; Gómez-Fernández et al., 2012; Tomás et al., 2012).

The aims of the present study were to evaluate the holding time prior to cryopreservation at 15 °C (2, 8 and 24 h), thawing rate (37 °C for 20 s or 70 °C for 8 s) and post-thawing incubation temperature (37 °C or 15 °C) on sperm quality after the freezing–thawing processes.

2. Material and methods

2.1. Reagents and media

Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO, USA), with purified water (18 MΩ cm; Automatic GR Wasserlab, Spain). Orvus ES Paste is marketed as Equex STM, Nova Chemical Sales Inc., Scituate, MA, USA. The basic medium used for sperm extension was Beltsville Thawing Solution (BTS, composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$, and 3.35 mM EDTA; Johnson, 1988), containing kanamycin sulphate (50 mg/mL). The basic extender used for sperm freezing was lactose egg yolk (LEY) (80% [v/v] 310 mM β-lactose, 20% [v/v] egg yolk, 100 μg/mL kanamycin sulphate; pH 6.2 and 330 ± 5 mOsm/kg).

2.2. Collection of sperm

The sperm-rich ejaculate fractions from three sexually mature crossbreed boars (Pietrain × Large White; Prosepor S.A., Segovia, Spain) were collected once a week for five consecutive weeks using the gloved-hand method. The boars with ages ranging from 1.5 to 2 years were housed in climate-controlled individual pens (15–25 °C) with a total of 16 h of light per day, were fed a commercial diet in accordance with the guidelines for the nutritional requirements for adult boars and received water *ad libitum*.

After collection, the semen samples were diluted in BTS (1:1, v/v), transferred to 50 mL plastic tubes, cooled to 15 °C, packaged in insulated containers and sent (in under 1 h) to the laboratory (Pig Research Centre, Instituto Tecnológico Agrario de Castilla y León, Spain) for cryopreservation.

After the laboratory received the samples, conventional semen characteristics were evaluated using standard laboratory techniques. Only ejaculates with ≥200×10^6 sperm/mL, ≥85% sperm with normal morphology, and with ≥75% and ≥80% of motile and viable spermatozoa, respectively, were selected for the study. The ejaculates were pooled before centrifugation and cryopreservation to reduce the variability due to the effect of the boar.

2.3. Freezing–thawing procedure

Spermatozoa were cryopreserved using the straw-freezing procedure described by Westendorf et al. (1975) and modified by Thurston et al. (2001) and Carvajal et al. (2004). At the laboratory, extended sperm-rich fractions were pooled and centrifuged at 2400 × *g* for 3 min at 15 °C (Universal 320 R, Hettich Zentrifugen, Germany; Carvajal et al., 2004), and the pellet was diluted in LEY to a concentration of 1.5×10^9 cells/mL. After further cooling to 5 °C within 120 min, diluted spermatozoa were re-suspended in LEY-glycerol-Orvus Es Paste (LEYGO) extender (92.5% LEY, 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass] and 6% glycerol [v/v]; pH 6.2, and 1650 ± 15 mOsm/kg) to yield a final concentration of 1×10^9 cells/mL. The resuspended and cooled spermatozoa were packed into 0.5 mL PVC-French straws (Minitüb, Tiefenbach, Germany), which were frozen using a controlled-rate freezer (IceCube 14S; Minitüb) as follows: from 5 to −5 °C at a rate of 6 °C/min, from −5 to −80 °C at 40 °C/min, held for 30 s at −80 °C, then cooled at 70 °C/min to −150 °C and plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for at least two weeks before thawing. Thawing of straws was done in a circulating water bath at 37 °C for 20 s and 70 °C for 8 s. Thawed sperm samples from three