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Research paper

Effect of semen washing on thawed ram spermatozoa subjected to a four hour post-thawing thermal evaluation test

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ABSTRACT

This study was conducted to evaluate sperm motility and qualitative characteristics of ram spermatozoa cryopreserved in the presence or absence of seminal plasma under controlled condition and subjected to a 4 h post-thawing thermal evaluation test. The aim being that exposing these frozen-thawed sperm to thermal resistance test will provide useful information on their survivability in the female genital tract towards fertilizing the ovum. Briefly, ejaculates from 5 males (5years) were collected by artificial vagina, and split into two aliquots. One aliquot was diluted (1:5) in Tris-citric acid-glucose (TCG) solution and washed twice by centrifugation at 600 × *g* for 10 min, while the other aliquot was kept unwashed. Thereafter, washed and unwashed sperm were extended in a TCG-based media containing 5% glycerol and 15% powdered egg yolk (PEY) and frozen. Frozen-thawed sperm were thawed and incubated at 37 °C for 4 h. Sperm motility characteristics was evaluated by CASA while plasma membrane integrity (SYBR-14/PI), acrosome integrity (PE-PNA), mitochondria activity (Mitotracker deep red) and reactive oxygen species (H_2DCFDA) were analysed by flow cytometry. Sperm nucleoprotein integrity assessment was done by the determination of overall levels of sperm-head disulfide bonds via spectrophotometric analysis of free-cysteine radicals' levels. Unwashed samples generally showed better results than washed samples as regards to sperm motility characteristics irrespective of incubation. The post-thawing incubation had a significant ($P < 0.05$) effect on acrosome integrity and mitochondria functionality irrespective of sperm treatment. This study demonstrates that the presence of seminal plasma prior to cryopreservation was beneficial in maintaining post thawed sperm motility, and as such, could be useful for ex situ ram sperm preservation towards its use for artificial insemination.

1. Introduction

The use of semen cryopreservation in assisted reproductive techniques (ART) has become an indispensable tool for genetic improvement towards breeding management in sheep industry (Anel et al., 2006). However, cryopreservation adversely impairs ram semen quality by altering sperm function (Ledesma et al., 2016). This may be due to spermatozoa sensitivity to extreme temperature changes during cooling, freezing and thawing, invariably leading to a reduction in motility, viability, mitochondria membrane integrity, increase in reactive oxygen species production and chromatin damage (Bailey et al., 2003; Said et al., 2010). These alterations may affect the post thawing fertilizing capabilities as a result of reduced survivability of the frozen thawed spermatozoa (Salamon and Maxwell, 1995; Ledesma et al., 2016).

Although, laparoscopic artificial insemination (AI) has yielded acceptable results (Naqvi et al., 2001), its use is usually sophisticated and cost effective, making it routinely inappropriate due to the extensive nature of the sheep industry. Nevertheless, there is still a wide variability with cryopreserved semen resulting to frequently low fertility rates (Bag et al., 2004). This is notably observed when cervical AI is performed, leaving substantive period of time for spermatozoa to migrate to the oviduct thus impairing sperm survivability (Druart et al., 2009). Therefore, maintaining the sperm quality of frozen-thawed spermatozoa for an extended period of time is of utmost importance.

However, several researchers on different species have reported that the longevity of frozen thawed spermatozoa during *in vitro* incubation is low due to the various alterations in sperm membrane during cryopreservation (Gadea et al., 2005; Domınguez-Rebolledo et al., 2009; Mata-Campuzano et al., 2012). Therefore, many attempts to improve the quality of frozen-thawed semen in various species have been reported; one such method is the removal of seminal plasma from the ejaculate by centrifugation (semen washing) prior to freezing (Peterson et al., 2007; Webb and Dean, 2009). Despite studies reporting the beneficial importance of seminal plasma prior to cryopreservation increased sperm resistance to cold shock and reducing cryocapacitation

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(Barrios et al., 2005; Fernandez-Juan et al., 2006), others have also demonstrated that removing seminal plasma was necessary to increase motility, membrane integrity and fertility after freeze-thawing (Moore et al., 2005; Maxwell et al., 2007).

Nevertheless, the necessity to eliminate the seminal plasma or not in ram sperm cryopreservation is still an ongoing issue. One reason for this discrepancy may be due to the complexity and variance of seminal plasma amongst species, breeds, donor age and or seasons (Mandiki et al., 1998; Muiño-Blanco et al., 2008; Leahy et al., 2010). However, a recent work in our laboratory has demonstrated that the removal of seminal plasma (washing by centrifugation) improved the sperm kinematics and survival of frozen-thawed ram sperm (Garcia, 2014). Despite the main aim- being to establish a semen bank for these endangered native Catalonian eed of rams (*Aranesa* and *Xisqueta*) under controlled conditions, information on sperm motility and qualitative characteristics during post-thawing incubation regarding the various sperm treatments prior to freezing is of preliminary importance. Therefore, exposing these frozen-thawed sperm to a thermal resistance test may be a useful technique in assessing their probability for survival in the female genital tract and capacitation status towards fertilizing the ovum (Aisen et al., 2000).

Therefore, in this present work, we tested the resilience of frozen-thawed sperm from washed and unwashed sperm samples during a 4-h incubation period. The objective was to determine the sperm physiological changes and qualitative characteristics by assessing their kinematic parameters, viability, nucleoprotein integrity, plasma membrane integrity, acrosome membrane integrity, mitochondrial function and reactive oxygen species (ROS) production at post thaw, and after a 4-h post-thawing incubation period. We aim at providing some basic information as regards to ram sperm processes for cryopreservation prior to testing its usefulness for further application.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, all reagents were acquired from Sigma Chemical Co. (St. Louis, Mo, USA). Fluorescence probes and analysis kits (LIVE/DEAD® sperm viability kit (L-7011; SYBR-14 and PI) and Mitotracker deep red (M22426) were purchased from Invitrogen (Barcelona, Spain). All fluorochrome solutions were kept in the dark at −20 °C. The freezing extender was prepared in our laboratory using powdered egg yolk as described by Palomo et al. (2017) obtained from NIVE (Nunspeet Holland Eiproducten). Incubation media used in this study was a modified phosphate buffer solution (PBS) (supplemented with 36 μg/mL pyruvate and 0.5 mg/mL BSA) with osmolarity of 280–300 mOsm and pH 7.3-7.4.

2.2. Animals and sample collection

Five adult male rams of *Aranesa* and *Xisqueta* breeds (aged 5 years) were used in this study. They were maintained under an intensive management system in Institute farm (IRTA, Caldes de Montbui, Barcelona, Spain) where semen collection and initial assessment was carried out. Semen was collected twice weekly with two ejaculates per collection from each male via artificial vagina with a total of six replications. Prior to collection, the preputial hairs were shaved and the orifice was washed with clean water and dried to prevent contamination of samples. All ejaculates were collected during the breeding season and maintained in water bath at 37 °C following initial analysis. Volume, mass motility, progressive motility and concentration were accessed immediately after collection through conventional methods. Only ejaculates of good quality were used and frozen (mass motility: ≥4; sperm concentration: ≥2500 × 10^6 sperm/ml: normal sperm morphology ≥70%).

2.3. Cryopreservation of semen

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution as defined by Salamon and Maxwell (2000), consisting of Tris (0.3 M), citric acid anhydrous (94.7 mM), and D(+)-glucose (27.75 mM). This solution was adjusted to a pH of 7.25 ± 0.05 and osmolarity 333 ± 2.80 mOsm. Thereafter, glycerol (5% v/v, final concentration) and antibiotics (1000 UI/mL sodium penicillin and 1 mg/mL streptomycin sulfate) were added to the solution having a final pH 7.0–7.17 and 1327 ± 234 mOsm. Powdered (PEY) egg yolk was then added to a final concentration of 15% as described by Palomo et al. (2017). The first and second ejaculates from each male were mixed together following no differences in the semen characteristics and divided into two equal samples. One sample was washed twice by dilution (1:5) in TGC by centrifugation at 600 X g for 10 min to remove the seminal plasma while the other sample was kept unwashed. Thereafter, the supernatant was carefully removed from the washed samples leaving the resulting sediment. All samples (washed and unwashed) was then diluted with the freezing extender (1:4). All extended samples were held for equilibration for 4 h at 5 °C. After cooling for 4 h (pre-freezing), the extended semen was packed into 0.25 ml plastic straws (IMV Technologies, L'Aigle, Cedex, France) at a final concentration of 400 × 10^6 sperm/ml and sealed with polyvinyl alcohol. All straws were kept in liquid nitrogen vapor (5 cm above the nitrogen level) for 10 min before been plunged into the liquid nitrogen and stored.

2.4. Sperm thermal evaluation test

Thermal stress test was carried out to verify the longevity of thawed semen samples. Two straws per treatment (wash and unwashed) per replicate were thawed and diluted in the modified PBS to 40 × 10^6 sperm/ml. Thereafter, samples were incubated at 37 °C in a dry bath in the dark. Evaluation on all post thawed parameters in this study was assessed immediately after thawing (0 h) and at 4 h of post-thawing incubation.

2.5. Semen evaluation

2.5.1. Motility assessment by CASA

Motion characteristics of pre-freezing (after 4 h of refrigeration) and post-thawed (0 h and 4 h of incubation) samples were assessed using the computer-assisted sperm analysis (CASA) system ISAS® (PROISER SL., Valencia, Spain). On analysis, all sperm samples were diluted (1:10) in PBS, and 5 μl drop of sperm suspension was placed on a slide and covered with a cover-slip (24 × 24 mm). Sperm motility was assessed at 37 °C at x200 magnification using a phase contrast microscope (Olympus BH-2, Japan). For each sample, more than three fields per drop were analysed and a minimum of 200 spermatozoa evaluated.

The percentage of total motility (TM), progressive motility (PM), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linearity coefficient (LIN = [VSL/VCL]x100, %), straightness coefficient (STR = [VSL/VAP]x100, %), lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) were evaluated. The settings used for the sperm image analyses were as follows: number of images (25/s), optical (Ph-), scale (20xOlympus), particle area (> 3 a < 70 micras^2), slow sperm (10–45 micras/s), average sperm (45–75 micras/s), rapid sperm (> 75 micras/s), progressive (80% STR). All diluents and materials used for sperm analysis were maintained at 37 °C.

2.5.2. Flow cytometry analysis

Flow cytometry was performed using the BD FACSCanto platform (BD Biosciences, USA), and data were analysed by BD FACS DIVA software (BD Biosciences, USA). Plasma and acrosomal membrane integrity as well as mitochondrial function were evaluated by flow

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