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Cryopreservation of donkey sperm using non-permeable cryoprotectants

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ABSTRACT

The aim of this study was to evaluate the effect of different concentrations of sucrose combined with bovine serum albumin (BSA), as non-permeable cryoprotectants, on donkey sperm parameters after cryopreservation, in comparison to a control extender containing glycerol. Semen from five Andalusian donkeys (n = 12) were centrifuged and resuspended with a commercial extender for equine sperm (Gent A, Minitube) adding 1% BSA and different concentrations (M, mol/l) of water-diluted sucrose: 0.05, 0.1, 0.25, 0.35 and 0.45. Thereafter, semen (n = 24) were diluted in the same base extender containing 0.25 M sucrose (S25) or glycerol (GLY, Gent B). Sperm were slowly cooled, filled in 0.5 ml straws and frozen in nitrogen vapours. Post-thaw samples were assessed for sperm motility, plasma membrane and DNA integrity and results were compared by ANOVA. In Experiment 1, sperm motility was significantly higher ($P < 0.001$) for S25 than the remaining treatments, and no differences were found for plasma membrane or DNA integrity. In Experiment 2, no differences were found between S25 or GLY for sperm motility and DNA integrity but plasma membrane integrity was significantly higher ($P < 0.05$) for S25. In conclusion, the extender with sucrose 0.25 M combined with BSA can be considered as an alternative to conventional extenders with glycerol for donkey sperm cryopreservation.

1. Introduction

Cryopreservation of donkey semen is an important tool to maintain the genetic diversity and preserve endangered species (Watson, 2000). Based on Food and Agriculture Organization of the United Nations (FAO) criteria, and according to the Spanish regulations (RD 698/2013), Andalusian donkey breed is in danger of extinction.

It is well known that freezing and thawing lead to cell death or sublethal (apoptotic) cryoinjury, mostly caused by osmotic stress and the toxicity due to unequal distribution of permeable cryoprotective agents (CPAs) on the sperm cell (Morris et al., 2007; Pena et al., 2011; Macías García et al., 2012; Wu et al., 2015). In this regard, donkey sperm seems to be sensitive to glycerol (Vidament et al., 2009), the most used permeable cryoprotectant for equine sperm preservation in the last 50 years (Hoffmann et al., 2011). Besides, its toxic effect has been shown to start as early as the pre-freezing process begins (Vidament et al., 2005; Oliveira et al., 2006; Vidament et al., 2009; Rota et al., 2012; Serres et al., 2014). A negative effect of the permeable CPAs in the uterus of the jenny has

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been also hypothesized (Vidament et al., 2009; Serres et al., 2014; Oliveira et al., 2016), as the post-insemination reaction is more dramatic in jennies than in mares when permeable CPAs are present (Vidament et al., 2009). Moreover, fertility using cryopreserved donkey sperm is also lower in jennies than in mares (Vidament et al., 2009).

Considering the sensitivity of donkey sperm to permeable CPAs (Vidament et al., 2009; Acha et al., 2015), and neither substitution nor dilution avoids completely their toxicity (Oliveira et al., 2006; Vidament et al., 2009; Serres et al., 2014), non-permeable agents such as sucrose-based extenders combined with bovine serum albumin (BSA) could be an alternative for donkey sperm cryopreservation. This alternative has obtained quite satisfactory results in sperm motility, plasma membrane, chromatin integrity and fertility in human (Isachenko et al., 2003; Isachenko et al., 2004), canine (Sánchez et al., 2011), fish (Merino et al., 2011) and wild ruminant (Pradiee et al., 2015) after sperm cryopreservation.

Therefore, the aims of this study were to: 1) evaluate the effect of different concentrations of sucrose-based extenders combined with BSA on post-thaw donkey sperm parameters: motility, plasma membrane and DNA integrity; 2) compare the selected sucrose-based extender with a commercial freezing extender containing glycerol.

2. Material and methods

2.1. Semen collection and evaluation

A total of five healthy Andalusian donkeys, aged from 3 to 19 years were used as semen donors. The jackasses were housed in paddocks at “Centro Rural Malpica” (Palma del Rio, Cordoba, Spain) and the feeding consisted of water “ad libitum”, teff hay and oats. Semen was collected during the breeding season once or twice a week using a Missouri artificial vagina in the presence of a jenny in estrus. Ejaculates were assessed for sperm volume, concentration, morphology and motility before freezing as described by Dorado et al. (2013). All the experiments were approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

2.2. Freezing and thawing

Immediately after collection, semen was extended 1:1 (v:v) with INRA-96 (IMV Technologies, L’Aigle, France) and divided into aliquots. Then, each aliquot was centrifuged 7 min at 400 × *g* (22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). Thereafter, the supernatant was removed and the sperm pellet from each aliquot was re-suspended with its corresponding freezing extender (as described in 2.4 Experimental design) to reach a final sperm concentration of 200×10^6 spermatozoa/ml. Samples were equilibrated 10 min at room temperature (22 °C) and then slowly cooled to 5 °C into an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA) for 2 h. After that, sperm was loaded in 0.5-ml straws and frozen in liquid nitrogen (LN_2) vapours, 2.5 cm above the surface for 5 min, as described by Ortiz et al. (2015). Finally, each straw was plunged into LN_2 and stored for at least 24 h in a tank. A randomly selected straw from each treatment was thawed in a water bath at 37 °C for 30 s.

2.3. Post-thaw sperm evaluation

Thawed sperm were diluted to a final concentration of 25×10^6 sperm/ml with INRA-96 and sperm parameters were analysed as follows:

Total and progressive sperm motility were objectively evaluated using the Sperm Class Analyzer (SCA v.5.4.0.0; Microptic S.L., Barcelona, Spain) system. The SCA settings were previously described by Ortiz et al. (2015). Three drops, and two microscopic fields per drop, were analysed in each semen sample obtaining kinetic parameters: total and progressive motility (TM and PM, %), curvilinear velocity (VCL, μm s^{-1}), straight line velocity (VSL, μm s^{-1}); average path velocity (VAP, μm s^{-1}), linearity (LIN, VSL/VCL, %), straightness (STR, VSL/VAP, %), wobble (WOB, VAP/VCL, %), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm).

Plasma membrane integrity was assessed using the VitalTest (Halotech DNA SL, Madrid, Spain) as described by Dorado et al. (2014) and sperm with intact plasma membrane were recorded (PMI, %).

Sperm DNA integrity was assessed with the sperm chromatin structure assay (SCSA) using a FACScan Flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Two aliquots of 400 μl and 25×10^6 spermatozoa/ml were saved from each treatment. Those aliquots were kept in Eppendorf tubes and stored at −18 °C right after thawing (T0) and after four hours of incubation at 37 °C (T4). Chromatin integrity was assessed in each sample (T0 and T4) as previously described by Salazar et al. (2011). Around 5000 cells were studied, and the percentage of cells outside the main population, with single-stranded DNA was recorded (COMP-αt, %).

2.4. Experimental design

2.4.1. Experiment 1: comparison of different sucrose-based extenders for donkey semen cryopreservation

Four animals and three ejaculates per donkey (n = 12) were used in this experiment. A commercial extender for equine semen cooling without permeable cryoprotectants, which egg yolk and antibiotics (Gent A, Minitüb, Tiefenbach, Germany) and adding