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Effect of single-layer centrifugation or washing on frozen–thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?

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ABSTRACT

The aims of this study were to determine the sperm quality of frozen–thawed donkey sperm samples after single-layer centrifugation (SLC) using Androcoll-E in comparison to sperm washing or no centrifugation and to determine if the effect on the sperm quality after SLC or sperm washing depends on the quality of the sample. Frozen–thawed sperm samples from Andalusian donkeys were divided into three aliquots, and they were processed using three different techniques after thawing: uncentrifuged diluted control (UDC), sperm washing (SW), and SLC. Afterward, sperm quality index was estimated by integrating all parameters (total and progressive sperm motility, membrane integrity, and DNA fragmentation) in a single value. The relationship between the sperm quality of thawed UDC samples and the effect on sperm parameters in SW and SLC-selected samples was assessed. Sperm quality index was significantly higher ($P < 0.001$) in SLC (0.8 ± 0.0) samples than that in UDC (0.6 ± 0.0) and SW (0.6 ± 0.0) samples, regardless of the sperm quality index after thawing of the sperm sample. In conclusion, SLC of frozen–thawed donkey spermatozoa using Androcoll-E-Small can be a suitable procedure for selecting frozen–thawed donkey sperm with better quality, in particular in those samples where an improvement in motility is needed.

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1. Introduction

Spanish donkey breeds are in danger of extinction; considering the importance of biodiversity and of preservation of domestic species resources, the creation of genetic banks for these donkey breeds is needed [1]. However, freezing and thawing causes major damage to the spermatozoa, particularly to their plasma and organelle membranes [2–4]. The most important factors causing cryoinjury are considered to be the osmotic stress caused by dehydration of the extender during freezing and thawing [5,6] and the toxicity caused by unequal distribution of cryoprotectants [7].

Different strategies have been proposed to improve postthaw sperm quality, including dilution or removing freezing extenders by simple sperm washing (SW) to reduce the concentration of cryoprotectant in the semen sample. It has been hypothesized that glycerol, the cryoprotectant most commonly used for horse semen cryopreservation, may be toxic for donkey jack sperm [8] or exert a negative effect on donkey jenny fertility [9]. These

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facts could be responsible for the disappointing results of artificial insemination (AI) with frozen–thawed donkey sperm [1]. However, when several studies investigated this phenomenon, glycerol could not be confirmed as responsible for either toxicity of donkey sperm [10] or female uterus inflammation [9,11]. Thus, that hypothesis currently remains unclear.

Additionally, replacement of freezing extenders with seminal plasma by dilution or simple SW after thawing has recently been proposed for processing donkey sperm straws before AI [1]. However, in that study, better postthaw sperm motility was shown when frozen–thawed donkey sperm was reextended with semen extender rather than with seminal plasma.

An alternative approach to improve the quality of frozen–thawed sperm doses and to remove the cryoprotectant used would be to select those spermatozoa that are most likely to achieve fertilization from the rest of the semen sample [12]. In this sense, centrifugation of stallion sperm through a species-specific single layer of silica colloid (single-layer centrifugation [SLC]), called Androcoll-E, has been shown to select sperm with better motility, normal morphology, membrane integrity, and intact chromatin from the rest of the ejaculate [13–15], and it also increases pregnancy rates [16]. However, to our knowledge, no studies comparing redilution, SLC, and SW have been performed in donkey frozen–thawed sperm samples.

The aims of this study were to (1) determine if SLC using Androcoll-E on frozen–thawed donkey sperm samples could select high-quality sperm in comparison to SW or no centrifugation, (2) determine if the effect on the sperm quality after SLC or SW depends on the quality of the sample.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation. Six healthy, mature, Andalusian donkeys, aged from 6 to 15 years, were used as semen donors. The jackasses were owned by “Donkey's House Foundation” (Rute, Córdoba, Spain) and were housed in individual paddocks placed at the Veterinary Teaching Hospital of the University of Córdoba (Spain). The feeding consisted of alfalfa hay and water “*ad libitum*.”

2.2. Semen collection

Semen was collected using a Missouri artificial vagina with an in-line gel filter (Minitüb, Tiefenbach, Germany) in the presence of a jenny in natural or induced estrus to stimulate copulatory activity. Semen was collected from each animal twice a week. Three ejaculates were collected from each donkey on different sampling occasions obtaining a total number of 18 ejaculates. Total and progressive sperm motility were objectively evaluated from fresh semen by using the Sperm Class Analyzer (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain) system as described later. Gel-free volume (mL) was measured in a graduated collector. Sperm concentration ($\times 10^6$ spermatozoa/mL) was assessed with a sperm photometer (Spermacue; Minitüb).

2.3. Sperm freezing and thawing

Immediately after collection, semen was extended 1:1 (v:v) in EquiPro (Minitube) and centrifuged 7 minutes at $400 \times g$. After that, the supernatant was removed and the sperm pellet was reextended in a commercial freezing medium with glycerol (Gent; Minitube) to a final concentration of 200×10^6 sperm/mL. Semen was slowly cooled to 5 °C within 2 hours, loaded in 0.5-mL plastic straws, and frozen horizontally in racks placed 2.5 cm above the surface of the liquid nitrogen for 5 minutes. The straws were then directly plunged in liquid nitrogen, and after at least 1 week of storage, they were thawed in a water bath at 37 °C for 30 seconds.

2.4. Postthawing sperm processing

2.4.1. Uncentrifuged diluted control

One semen straw was thawed and directly diluted with INRA 96 (IMV Technologies, L'Aigle, France) to a final concentration of 25 million sperm/mL. This treatment was considered as uncentrifuged diluted control (UDC). Postthaw sperm parameters were analyzed as described in the following.

2.4.2. Sperm washing

Immediately after thawing, one semen straw was extended at the 1:1 ratio and centrifuged at $400 \times g$ for 7 minutes. The supernatant was removed, and the sperm pellet was resuspended to a final concentration of 25 million sperm/mL for sperm analysis.

2.5. Single-layer centrifugation

Sperm selection was carried out on frozen–thawed semen samples using Androcoll-E-Small which is a glycidoxypropyltrimethoxysilane-coated silica colloid. Androcoll-E-Small was allowed to equilibrate to room temperature (22 °C) for 30 minutes before use. Then, 2 mL of frozen–thawed semen (three straws extended until a final concentration of $50–100 \times 10^6$ sperm/mL) was carefully layered on the top of 4 mL of Androcoll-E-Small located in a 15-mL corning tube. The suspension was centrifuged at $300 \times g$ for 20 minutes. The supernatant was removed, and the sperm pellet was recovered and transferred to a clean tube containing extender. The sperm concentration of the resuspended sperm pellet in 1 mL of extender was measured using the SCA. After that, the sperm concentration of SLC-selected samples was adjusted to a final concentration of 25 million sperm/mL, and then, sperm parameters were analyzed as described in the following. The yield of selected spermatozoa was calculated according to the following formula: yield = (number of spermatozoa* in sperm pellet/number of spermatozoa* in initial load) $\times$ 100; *yield was separately calculated for total, motile, progressively motile, intact membrane, and unfragmented-DNA spermatozoa.

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