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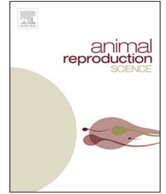

Stallion sperm selection prior to freezing using a modified colloid swim-up procedure without centrifugation

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ABSTRACT

The aims of this study were to: 1) develop a new method for stallion sperm selection using a modified swim-up procedure through a colloid and 2) evaluate its impact in good quality ejaculates from bad freezers in comparison to methods involving centrifugation such as single layer centrifugation and sperm washing. Ejaculates were processed before freezing using three different procedures: sperm washing (SW), colloid single layer centrifugation (SLC) and a modified colloid swim-up (SU). After semen processing, sperm recovery rates were measured and sperm were frozen. Post-thaw sperm motility (assessed by computer-assisted sperm analysis), normal forms and plasma membrane integrity (evaluated under bright-field and fluorescence microscopy respectively), and DNA fragmentation (assessed by the Sperm-Halomax kit) were compared between treatments. Sperm recovery rates were similar between SU and SLC but lower than SW. Sperm motility after thawing was lower in SU in comparison to SLC and SW, maybe due to the incomplete removal of seminal plasma before freezing. Sperm DNA fragmentation was lower in SU and SLC selection methods, particularly in SLC selected samples during the first 6 h of incubation. The remaining sperm parameters assessed were similar among treatments. In conclusion, SLC is more suitable than SW and SU to process stallion semen prior to freezing, in particular when sperm DNA damage is suspected. Further studies are needed in order to determine the potential benefits of SU in samples where centrifugation is not necessary, such as epididymal sperm, ejaculate fractioning or post-thaw semen samples.

1. Introduction

Cryopreservation causes major damage to equine spermatozoa which may result in low sperm quality after thawing (Watson, 2000). Moreover, post-thaw semen quality and sperm freezability vary among stallions. Due to this fact, stallions have been classified as good, average or poor freezers according to the ability of their spermatozoa to withstand the freezing and thawing process (Loomis and Graham, 2008). A number of strategies have been developed to improve freezing and thawing procedures in equine species (Salazar et al., 2011; Contri et al., 2012; Legha and Pal, 2012) including the selection of those spermatozoa that are most likely to

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achieve fertilization from the rest of the sperm population (Morrell et al., 2008). In this regard, colloid single-layer centrifugation (SLC) has been widely used to select good quality sperm for different species, including horses (Johannisson et al., 2009; Gutiérrez-Cepeda et al., 2011; Morrell et al., 2011).

Sperm selection is a particularly important tool to select sperm from poor quality semen samples after thawing (García et al., 2009; Ortiz et al., 2015a) but there is little information about the impact of sperm selection before freezing. Hoogewijs et al. (2011) showed that SLC prior to freezing improved post-thaw sperm quality of stallion with sub-standard sperm parameters. Some of these stallions had poor quality ejaculates before freezing.

In equine species, sperm washing (SW) prior to freezing is important to remove seminal plasma and to obtain a cleaner sample with an appropriate cell concentration (Sieme et al., 2004). Both SW and SLC include centrifugation of the semen sample in their protocols. In this regard, semen processing methods, including centrifugation, may produce cell damage at the end of the process (Knop et al., 2005; Sieme et al., 2006; Álvarez-Rodríguez et al., 2016).

In this study a new method for stallion sperm section without centrifugation using a modified swim-up procedure through a colloid is proposed. The sperm migration or swim-up method is commonly used for the isolation of highly motile spermatozoa from the ejaculate (Rodriguez-Martinez et al., 1997). However, the standard swim-up procedure involves centrifugation steps that induce a decline in the motility of spermatozoa (Alvarez et al., 1993). A swim-up method, without centrifugation, was developed by García-López et al. (1996) for the isolation of motile and highly viable ram spermatozoa. In order to optimize those results, an alternative procedure was developed by Dorado et al. (2016) for dog sperm selection before cooling using a column of colloid between the semen sample and the migration medium. It may help to select not only motile sperm but also viable sperm with normal morphology and intact DNA without centrifugation.

Therefore, the aim of this study was to develop a new method for stallion sperm selection using a modified swim-up procedure through a colloid and evaluate its impact in good quality ejaculates from bad freezers in comparison to methods involving centrifugation such as single layer centrifugation and sperm washing.

2. Materials and methods

2.1. Animals

Six healthy stallions aged from 6 to 15 and with known fertility were used as semen donors. Animals were owned by FESCCR-Ministry of Defense and were housed in individual paddocks placed at the equine breeding services of the Spanish Army located in Avila, Spain (40.66°N, 4.70°W). The feeding consisted of commercial concentrate, alfalfa hay and water “ad libitum”. Stallions were classified as “bad freezers” according to the criteria of Loomis and Graham (2008). All the ejaculates used in this study showed a post-thaw progressive sperm motility < 30%. These animals failed to produce better sperm motility after thawing using others freezing protocols.

2.2. Semen collection

Extragonadal sperm reserves were depleted before the animals were involved in a regular basis of semen collection during the breeding season (abstinence period < 7 days). Semen was collected from the stallion in the presence of a mare in estrus to induce sexual activity after mounting a phantom using a Missouri artificial vagina with an in-line gel filter. Two ejaculates were collected from each stallion twice a week on different sampling occasions, obtaining a total number of twelve ejaculates. Immediately after collection, gel-free volume (V, ml) was measured in a graduated collector and sperm concentration (C, $x10^6$ sperm/ml) using a sperm photometer (Spermacue®; Minitüb, Tiefenbach, Germany). The remaining sperm parameters were assessed as follows.

2.3. Semen assessment

2.3.1. Computer-assisted sperm motility analysis (CASA)

Sperm motility was evaluated by CASA using the Sperm Class Analyzer system (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain). Features were described previously (Ortiz et al., 2014). An aliquot of each semen sample was extended with INRA96 (IMV Technologies, L'Aigle, France) to a final concentration of 25×10^6 sperm/ml and then incubated at 37 °C for 5 min. After that, three 5 μl-drops of each diluted semen sample were placed in a D4C20 chamber (Projectes i Serveis R + D S.L., Valencia, Spain). Two randomly chosen microscopic fields per drop were analysed in each semen sample. Total (TM, %) and progressive sperm motility (PM, %) were recorded.

2.3.2. Sperm morphology

Sperm morphology was examined by light microscopy evaluation on smears stained with Diff-Quick® (Medion Diagnostics AG, Düdingen, Switzerland), increasing the time of each step to 1 min as described previously (Hidalgo et al., 2006). At least 200 sperm per slide were counted to determine the percentage of sperm with normal forms (NF, %).

2.3.3. Plasma membrane integrity

Sperm membrane integrity was assessed using the double stain propidium iodide (PI) with acridine orange (AO) from the Vital-Test kit (Halotech DNA SL, Madrid, Spain), following the manufacturer instructions. At least 200 sperm were counted, considering

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