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# Colloid centrifugation of fresh stallion semen before cryopreservation decreased microorganism load of frozen-thawed semen without affecting seminal kinetics

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ABSTRACT

Freezability of equine semen may be influenced by microorganism population of semen. The objective of this study was to verify the effect of single-layer density gradient centrifugation (SLC) of fresh semen before cryopreservation on semen's microbial load (ML) and sperm cells kinetics after freezing-thawing. For that, one ejaculate was collected from 20 healthy stallions and split into control (C) samples (cryopreserved without previous SLC) and SLC samples (subjected to SLC). Semen cryopreservation was performed according to the same protocol in both groups. Microbial load of each microorganism species and total microbial load (TML) expressed in colony-forming units (CFU/mL) as well as frozen-thawed sperm kinetics were assessed in both groups. Additional analysis of the TML was performed, subdividing the frozen-thawed samples in "suitable" (total motility $\geq$ 30%) and "unsuitable" (total motility $<$ 30%) semen for freezing programs, and comparing the C and SLC groups within these subpopulations. After thawing, SLC samples had less ($P < 0.05$) TML ($88.65 \times 10^2 \pm 83.8 \times 10^2$ CFU/mL) than C samples ($155.69 \times 10^2 \pm 48.85 \times 10^2$ CFU/mL), mainly due to a reduction of *Enterococcus* spp. and *Bacillus* spp. A relationship between post-thaw motility and SLC effect on ML was noted, as only in samples with more than 30% total motility was ML reduced ($P < 0.05$) by SLC (from $51.33 \times 10^2 \pm 33.26 \times 10^2$ CFU/mL to $26.68 \times 10^2 \pm 12.39 \times 10^2$ CFU/mL in "suitable" frozen-thawed semen vs. $240.90 \times 10^2 \pm 498.20 \times 10^2$ to $139.30 \times 10^2 \pm 290.30 \times 10^2$ CFU/mL in "unsuitable" frozen-thawed semen). The effect of SLC on kinetics of frozen-thawed sperm cells was negligible.



## 1. Introduction

It has been estimated that 20% of stallions produce semen that freezes well, 60% semen that freezes acceptably, and 20% of the stallions have semen that freezes poorly [1,2]. The mechanisms underlying the differences in cryosensitivity between individuals are largely unknown [2], with those differences attributed to genetic factors [2,3], type of cryoprotectant utilized [3,4], and other nongenetic factors not yet elucidated [2].

Bacterial contamination is known to affect semen during cold storage [5]. Frozen-thawed semen contains bacteria [6–8], and Hoogewijs et al. [9] hypothesized that a reduction in bacterial contamination would improve the

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quality of frozen-thawed semen. However, influence of bacteria in the quality of frozen-thawed equine semen has not been proven.

In humans, semen contaminated with bacteria can compromise the outcome of assisted reproductive techniques or promote infection of the female genital tract [10]. In horses, it has been recognized that bacteria present in the stallion external genitalia and semen can be transmitted to mares [11,12] and may lead to uterine pathology and infertility, especially in susceptible mares [6,12,13]. Consequently, antibiotics are routinely added to semen extenders [14], but some may have an adverse effect on sperm quality and, in general, are ineffective below 15 °C [5]. Additionally, even small concentrations of antibiotics can result in antibiotic resistance [15,16], and, thus, its prophylactic utilization in semen extenders raises concerns.

Density gradient centrifugation effectively reduced bacterial contamination of human semen [10,17], and bacterial contamination in boar semen was decreased by single-layer density gradient centrifugation (SLC) using the species-specific colloid Androcoll-P [14]. Recently, SLC through Androcoll-E greatly reduced the bacterial load in stallion semen "spiked" with known concentrations of specific microorganisms [16]. In addition, improvement of seminal characteristics of refrigerated stallions' ejaculates [18,19] and of frozen-thawed sperm after SLC were reported [20,21], and Hoogewijs et al. [9] noted an overall increase in sperm quality in some equine ejaculates subjected to SLC before cryopreservation.

The objectives of this study were (1) to verify if SLC of fresh semen through Androcoll-E before cryopreservation decreases microbial content over non-SLC–treated samples; and (2) to assess post-thaw semen characteristics of SLC-treated and nontreated samples of the same ejaculates.

## 2. Materials and methods

### 2.1. Animals and experimental design

During the breeding season (February to August) of 2012 and 2013, 20 healthy stallions of different breeds (12 Pure Breed Lusitano, 1 Belgian Warm Blood, 2 Thoroughbreds, 1 French Saddle, 2 Sorraia, and 2 Lusitano crossbreds) with ages ranging from 5 to 22 years were used for semen collection (1 ejaculate/stallion), processing, and cryopreservation. Progressive motility of all ejaculates was assessed subjectively by light microscopy (×200), placing a semen droplet in a prewarmed (37 °C) slide covered by a cover slip.

Semen was collected using a Missouri or an Institut National de la Recherché Agronomique (IMV Technologies, L'Aigle, France) artificial vagina. A sterile semen collection bottle was used in each collection, and a new internal liner (for the Institut National de la Recherché Agronomique artificial vagina) or a newly sanitized Missouri artificial vagina was utilized for each collection. A mare in estrous was used as mount.

### 2.2. Semen processing

The collected gel-free ejaculates were diluted to a concentration of $100 \times 10^6$ sperm cells/mL in prewarmed (37 °C) antibiotic-free EquiPro extender (Minitub Ibérica S.L., Tarragona, Spain) and split into two groups: standard centrifugation (C) and SLC.

Control centrifugation group (C): Extended ejaculates were centrifuged at 900× *g* for 10 minutes. After centrifugation, the supernatant was removed using a sterile Pasteur pipette.

Single-layer density gradient centrifugation group: Fifteen milliliters of Androcoll-E were poured into a 45-mL sterile falcon tube, and 15 mL of extended ejaculate containing $100 \times 10^6$ spermatozoa/mL were carefully pipetted on top of the Androcoll-E layer. After centrifugation at 600× *g* for 20 minutes, the supernatant (semen extender, seminal plasma, and colloid) was removed using a sterile Pasteur pipette. The total number of spermatozoa remaining in the pellet was calculated using a Neubauer chamber.

Cryopreservation: After centrifugation, the spermatozoa pellet obtained from both groups was cryopreserved as described by Alvarenga et al. [4]. Briefly, the pellet was resuspended in an egg-yolk–based freezing extender (Botucrio, Botupharma, Botucatu, Brazil), which contains amikacin, to a final concentration of $200 \times 10^6$sperm cells/mL, and 0.5 mL straws were filled with the extended semen. Straws were then equilibrated at 4 °C for 20 minutes and subsequently placed in a rack located 6 cm above the liquid nitrogen surface for 25 minutes, after which the straws were plunged into liquid nitrogen and stored in a nitrogen tank. Semen manipulation and straw filling were performed in a horizontal laminar flow chamber (IDL 48.H model, Labolan S.L., Navarra, Spain).

### 2.3. Post-thaw motility evaluation

Straws were thawed in a water bath at 37 °C for 1 minute and their content transferred into a sterile eppendorf vial preheated at 37 °C. A sample of each straw was collected with a sterile pipette into a sterile eppendorf vial and sent refrigerated to the microbiology laboratory within 6 hours of collection. To this point, all semen manipulation was done inside the horizontal laminar flow chamber (IDL 48.H model, Labolan S.L.).

Sperm kinetics of frozen-thawed samples (C and SLC) were evaluated using a computer-assisted sperm analysis system (ISAS; Proiser, Valencia, Spain). For that, a small aliquot of semen was collected with a sterile Pasteur pipette and gently pipetted into a multichambered semen slide (ISAS-D4C20, ISAS). Five fields per sample were analyzed for percentage of total motility (TM), average path velocity (VAP), curvilinear velocity (VSL), straightness index (STR), and percentage of rapid cells (RAP). The settings used for the computer-assisted semen analysis were as follows: 25 frames/s, cell size ≥4 μm and ≤75 μm, velocity of rapid cells greater than 90 μm/s, straightness 30%, and temperature 37 °C.

Loomis and Graham [22] classified frozen-thawed semen with ≥30% progressive motility as "suitable" for use in commercial programs, whereas samples with less than 30% motility rate were considered "unsuitable." Accordingly, we also analyzed the total microbial load (TML) in the SLC and C groups subdivided in "suitable" (≥30% TM) and "unsuitable" (<30% TM) frozen-thawed samples.