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# The impact of cushioned centrifugation protocols on semen quality of stallions

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**Abstract**

The objective was to determine if decreased cushion-fluid volume and increased sperm number during centrifugation, or if sperm concentration of extended semen following centrifugation, affected stallion sperm quality. Three ejaculates from each of three stallions were subjected to cushioned centrifugation (1,000*g* for 20 min). Cushion-fluid volume was set at 1 or 3.5 ml, and sperm number per centrifuge tube was set 1 billion or 3 billion. Following centrifugation, sperm pellets were resuspended in semen extender containing 20% seminal plasma (v/v) with sperm concentrations of 25 or 250 million/mL. Sperm recovery rate among centrifugation treatment groups was compared. Motion characteristics, plasma membrane intactness (SMI), and DNA quality (COMP$\alpha$t) of sperm were compared among treatment groups and uncentrifuged controls immediately following centrifugation (Time 0 h) and following 24 h of cooled storage (Time 24 h). Centrifugation treatment did not affect sperm recovery rate ($P > 0.05$). At Time 0 h, no differences in experimental end points were detected between cushion-fluid volumes tested ($P > 0.05$). Values for percent total sperm motility, percent progressive sperm motility, and track straightness were similar between sperm-number treatments subjected to centrifugation ($P > 0.05$). At Time 24 h, values for all experimental endpoints were similar between centrifugation treatments for cushion volume per tube, and between centrifugation treatments for sperm number per tube ($P > 0.05$). Centrifugation treatments and control treatments were similar for five of six variables tested ($P > 0.05$). Sperm storage concentrations of $25 \times 10^6$ and $250 \times 10^6$/mL yielded similar values for percent total sperm motility, percent progressive sperm motility, percent SMI, and percent COMP$\alpha$t ($P > 0.05$). A storage concentration of $250 \times 10^6$ sperm/ml yielded higher values for curvilinear velocity, and lower values for straightness, than all other groups ($P < 0.05$). In conclusion, centrifugation with as little as 1 ml of cushion fluid and a sperm number of up to $3 \times 10^9$ sperm in 50-ml conical-bottom centrifuge tubes had no detrimental effect on initial or cool-stored sperm quality. Additionally, storage of centrifuged sperm at a concentration of $250 \times 10^6$/mL with 20% seminal plasma (v/v) did not have a detrimental effect on percentages of motile or progressively motile sperm, or sperm DNA quality.


*Keywords:* Sperm; Centrifugation; Cushion; Sperm storage; Horse

## 1. Introduction

Centrifugation of stallion semen is frequently done in equine breeding programs to reduce seminal plasma concentration and/or increase sperm concentration in extended semen for insemination of mares. The procedure has particular relevance to processing of semen for cooled or frozen storage, or when using a low-dose insemination technique. Cushioned centrifugation of stallion semen has become an increasingly popular laboratory technique, as it maximizes sperm harvest without apparent injury to sperm [1–6].

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The medium typically used for cushioned centrifugation of stallion sperm contains a non-ionic iodinated compound, iodixanol. This iodinated medium was first reported for density-gradient cell fractionation [7–9], and has since been used as either a density gradient or as a cushion for centrifugation of sperm in various species, particular those producing ejaculates with relatively low sperm concentrations [1–6,10–13].

Despite the increasing application of cushioned centrifugation to stallion semen in clinical and research situations, the impacts of altered sperm number and cushion volume per standard centrifuge tube on post-centrifugation sperm quality has apparently not been reported. In addition, there is a paucity of information regarding the effect of sperm concentration in extended semen on sperm quality following cooled storage when seminal plasma concentration is held constant. The purpose of this study was to address the effects of these three features on stallion semen quality following cushioned centrifugation.

## 2. Materials and methods

### 2.1. Stallions and semen collection

Three ejaculates from each of three fertile American Quarter Horse stallions, aged 9 to 20 y, were used. The penis was washed with water and dried while erect, immediately before collection of semen. An ovariectomized mare in behavioral estrus was used for sexual stimulation and each stallion was mounted on a breeding phantom for semen collection, using an artificial vagina (Missouri Model AV, Nasco, Ft. Atkinson, WI, USA) fitted with a nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA). The artificial vagina was lubricated with 3 to 5 ml of sterile non-spermicidal lubricant (Priority Care, First Priority, Inc., Elgin, IL, USA) before the semen was collected. Sperm concentration was determined using an automated cell counter (NucleoCounter-SP100; Chemometec, A/S, Allerød, Denmark), as described [14].

### 2.2. Semen processing

As control treatments, aliquots of gel-free semen were subjected to simple dilution in semen extender (INRA96; IMV, L'Aigle, France) to $25 \times 10^6$ sperm/ml (where seminal plasma concentration ranged from 7.9 to 20.8% (v/v); Group SD25), or were diluted with INRA96 at a ratio of 4 parts extender: 1 part semen (Group 4:1; 20% seminal plasma [v/v]; final sperm concentration ranged from 28.8 to $83.7 \times 10^6$ sperm/ml). Group SD25 was used as an uncentrifuged control for evaluation of semen soon after semen was collected (Time 0 h), whereas Group SD25 and Group 4:1 were used as uncentrifuged controls for evaluation of semen following 24 h of cooled storage (Time 24 h). For centrifugation treatments, extended semen was subjected to cushioned centrifugation by layering two sperm quantities (1 or 3 billion sperm) over two cushion volumes (1 or 3.5 mL) before centrifugation in 50-mL conical-bottom centrifuge tubes. Semen was first loaded into centrifuge tubes and then extended to 40 ml with INRA96. Cushion fluid (Cushion Fluid, Minitüb, Tiefenbach, Germany) was layered under extended semen using an 18-ga 3.5-in spinal needle cut flat at the bevel. The resulting sperm concentrations were $25 \times 10^6$ or 75 million/mL in centrifuge tubes containing 1 billion or 3 billion sperm, respectively. These samples were centrifuged at 1,000*g* for 20 min, as described [5]. Supernatant in tubes containing 3.5-ml cushion was aspirated to approximately the 5-ml level, whereas supernatant in tubes containing 1-ml cushion was aspirated to the top of conical portion of the tube (approximately the 3.5-ml level). Following aspiration of the supernatant, the cushion fluid was aspirated from beneath the sperm pellet, using an 18-ga 3.5-in spinal needle cut flat at the bevel. Sperm pellets were resuspended in INRA96 extender containing 20% (v/v) fresh seminal plasma, and then adjusted to 25 or 250 million sperm/ml. Fresh seminal plasma was procured by centrifuging raw semen for 10 min at 2,000*g*. The supernatant (seminal plasma) was removed from the resulting sperm pellet and transferred to an all-plastic syringe fitted with 5.0- and 1.2-$\mu$m nylon filters (Cameo 30 N Syringe Filter, Nylon, 30 mm; Sigma-Aldrich, St. Louis, MO, USA) to removing any remaining sperm in the seminal plasma. Extended semen was subjected to immediate analysis, or packaged in 2-mL polypropylene tubes (Cryogenic vials; Corning Life Sciences, Lowell, MA, USA) with minimal air space, and then placed in a commercial semen-transport container (Equitainer I; Hamilton Research, Inc., South Hamilton, MA, USA) for cooling and storage for 24 h, followed by analysis.

Samples were evaluated for sperm recovery rate (percentage of sperm recovered following centrifugation) at Time 0 h, and for sperm motion characteristics, plasma membrane intactness, and sperm DNA quality at Time 0 h and Time 24 h.

### 2.3. Assessment of sperm quality characteristics

Sperm motion characteristics were assessed using a computerized sperm motility analyzer (IVOS Version