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Factors affecting sperm recovery rates and survival after centrifugation of equine semen

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Abstract

Conventional centrifugation protocols result in important sperm losses during removal of the supernatant. In this study, the effect of centrifugation force (400 or 900 x g), duration (5 or 10 min) and column height (20 or 40 mL) (Exp. 1); sperm concentration (25, 50 and 100 x 10^6/mL; Exp. 2) and centrifugation medium (EZ-Mixin CST, INRA96 or VMDZ; Exp. 3) on sperm recovery and survival after centrifugation and cooling and storage was evaluated. Overall, sperm survival was not affected by the combination of centrifugation protocol and cooling. Total sperm yield (TY) was highest after centrifugation for 10 min at 400 x g in 20-mL columns (95.6 ± 5 %) or 900 x g in 20- (99.2 ± 0.8 %) or 40-mL (91.4 ± 4.5 %) columns, and at 900 x g for 5 min in 20-mL columns (93.8 ± 8.9 %) ($P < 0.0001$). Total (TMY) and progressively motile (PMY) sperm yield followed a similar pattern ($P < 0.0001$). Sperm yields were not significantly different among samples centrifuged at different sperm concentrations. However, centrifugation at 100 x 10^6/mL resulted in significantly lower TY (83.8 ± 10.7 %) and TMY (81.7 ± 6.8 %) compared with non-centrifuged semen. Centrifugation in VMDZ resulted in significantly lower TMY (69.3 ± 22.6 %), PMY (63.5 ± 18.2 %), viable yield (60.9 ± 36.5 %) and survival of progressively motile sperm after cooling (21 ± 10.8 %) compared with non-centrifuged semen. In conclusion, centrifuging volumes of ≤ 20 mL minimized sperm losses with conventional protocols. With 40-mL columns, it may be recommended to increase the centrifugal force to 900 x g for 10 min and dilute the semen to a sperm concentration of 25 to 50 x 10^6 /mL in a milk- or fractionated milk-based medium. The semen extender VMDZ did not seem well suited for centrifugation of equine semen.

Keywords: Centrifugation, stallion, semen, viability, recovery

1. Introduction

Equine semen is routinely centrifuged prior to cryopreservation to concentrate sperm and minimize the adverse effects of seminal plasma on post-thaw motility [1,2]. Depending on the semen extender used, centrifugation and partial removal of seminal plasma prior to cooling may also be beneficial for sperm motility, and acrosome and DNA integrity, especially for stallions whose sperm suffer a significant decrease in motility when processed in a conventional manner by simple dilution of seminal plasma with semen extender [3-6]. Ejaculates with low sperm concentration require centrifugation to allow adequate dilution of semen for cooling [7].

In conventional centrifugation protocols, equine semen is diluted 1:1 (v:v) or to a sperm concentration of 50 $x10^6$ /mL in a milk-based semen extender for centrifugation. A 40-mL volume of extended semen is typically loaded into 50-mL conical tubes, and centrifuged at 400 to 600 x g for 10 to 15 min [7]. After centrifugation, 30 mL of the supernatant is removed, retaining 5 to 20 % of seminal plasma in the resuspended sample [7]. The final concentration of seminal plasma depends on the amount of semen extender added to the pellet. Around 20 to 25 % of sperm are lost with the supernatant during conventional centrifugation protocols [7,8], with losses of up to 46 % of sperm reported [9]. This results in an important reduction in the number of insemination doses available per ejaculate. A centrifugation protocol that improves sperm recovery, without damaging the cells, would result in a higher number of viable sperm available for cryopreservation or insemination.

Cushioned centrifugation in optically clear media has been reported to improve recovery rates without detrimental effects on sperm viability compared to conventional centrifugation protocols [10,11]. However, the improved recovery rates are likely to result from increased centrifugation duration (20 min) and forces (1000 x g) used during cushioned centrifugation [11]. In fact, better recovery rates were obtained after centrifugation in an opaque medium at 1000 x g for 20 min without an underlaying cushion compared with the addition of a cushion [11]. Use of a cushion to protect equine sperm against damage associated with close packing was previously suggested to be unnecessary [12]. Use of cushioned centrifugation increases the time and expenses associated with centrifuging equine semen. A simpler centrifugation protocol that improves recovery rates without damaging sperm and increasing processing time and expenses would be of benefit for the equine industry.

Sedimentation rate, and therefore sperm recovery, is determined by the centrifugal force and duration of centrifugation. Centrifugation duration and force are reciprocal, and total yield increases linearly as the product of duration x force increases until it reaches full sedimentation at 100 % [13,14]. Once full sedimentation is reached, viable and motile yields decrease as a consequence of cell damage in the pellet and the lack of further arrival of undamaged cells [13,14]. A particle also experiences a greater centrifugal force the further away it is from the axis of rotation. A shorter column height in a partially filled tube increases the minimum radial distance of the particles from the axis of rotation. Therefore, particles start to sediment at a higher gravitational field, have a reduced path length to travel, and sedimentation is quicker [13-15]. Sedimentation rate also depends on the difference in specific gravity between the cells and the surrounding medium, and the viscosity of the medium. This results in an increase in sedimentation rate as the density and viscosity of the medium decrease [13-15]. Initial sperm

concentration differs among ejaculates. Therefore, if an ejaculate is diluted with an equal volume of semen extender for centrifugation [7], semen is centrifuged at different sperm concentrations. While sedimentation rate can be affected by the initial concentration of the cell suspension [15], the effect of sperm concentration on recovery rates after centrifugation has not been critically evaluated. The objectives of this study were to determine the effect of two different centrifugal forces, durations, and column heights (volume), and three different sperm concentrations and media (semen extender) on sperm recovery rate and survival after centrifugation. Since centrifugation is often performed prior to cooling, delayed effects of centrifugation on sperm motility and viability after 24 h of cold storage at 4 to 8 °C were also evaluated.

2. Materials and Methods

2.1. Stallions and semen collection

Semen was collected from seven (Exp. 1 and 2) or five (Exp. 3) light breed adult stallions. Stallions 1 to 7 were used in Exp. 1. Stallions 8 to 14 were used in Exp. 2, while only stallions 8 to 12 were included in Exp. 3. Stallions were housed in individual pens supplemented with a pelleted ration and grass hay at the School of Animal Sciences or the School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana (Exp. 1) or Kansas State University, Manhattan, Kansas (Exp. 2 and 3). The stallions were teased with a mare in estrus and the penis was washed with warm water prior to semen collection. One ejaculate was collected from each stallion for each experiment with a Colorado (Exp. 1) or Missouri (Exp. 2 and 3) model artificial vagina over a phantom mare. Semen was obtained in February (Exp. 1),

August (Exp. 2) or September (Exp. 3) from sexually rested stallions. The internal temperature of the artificial vagina was adjusted at 45 to 48 °C, and sterile non-spermicidal lubricant (Priority Care, First Priority Inc., Elgin, IL, USA) was applied in the proximal one third of the artificial vagina immediately before collection. An in-line disposable nylon mesh gel filter (Animal Reproduction Systems, Chino, CA, USA) was used to exclude the gel fraction of the ejaculate. Immediately after collection, water was drained from the Colorado Model artificial vagina, the filter was removed, and the semen samples were transported to the laboratory for processing within 20 min of collection.

2.2. Evaluation of sperm concentration, motility and viability

Sperm concentration was evaluated using a Neubauer hemacytometer. While the method was not validated for repeatability in this study, the hemacytometer remains the gold standard for evaluation of sperm concentration [16,17]. Semen was diluted 1:100 in formalin buffered saline and spermatozoa were counted in the central grid of the hemacytometer. Both chambers of the hemacytometer were counted and averaged. If a difference greater than 10 % was found between chambers in the number of sperm counted, the hemacytometer was re-loaded and the sperm count was repeated. Sperm concentration was expressed in million per milliliter. During Experiment 1, sperm in the supernatant were counted using a 1:10 dilution and the sperm count was divided by 10.

Sperm motility was evaluated using a computer assisted sperm analyzer (Exp. 1: Sperm Vision, Minitube of America, Verona, WI, USA; Exp. 2 and 3: IVOS, Hamilton Thorn Research, Beverly, MA, USA). The settings of the instrument were: Frames acquired 45, frame rate 60 Hz,

minimum contrast 80, minimum cell size 3 pixels, straightness cut off 75 %, average path velocity cut off 50 μ/s, VAP cut off static cells 20 μ/s, cell intensity 106, static size gates 0.38 to 2.99, static intensity gates 0.77 to 1.4, and static elongation gates 12 to 97. Semen was placed in a 20-μL sperm analysis chamber (Hamilton Thorn Research) over the internal heated specimen stage at 37 °C. Mean percentages of total and progressive motility were assessed from 15 fields with a X 10 phase-contrast objective.

Membrane integrity or viability was evaluated with a fluorescent probe (SYBR14/PI, Live/Dead Kit, Molecular Probes, Eugene, OR, USA). First, 2 μL of a working solution of SYBR14 were added to 400 μL of semen. Semen was incubated for 10 min at 37 °C in the dark. Then, 2 μL of propidium iodide was added and semen was incubated for 10 min at 37 °C in the dark. Semen was evaluated using an epifluorescence microscope at high power (X 40) (Olympus B-Max 60, Olympus America, Inc., Melville, NY, USA). One hundred spermatozoa were classified as live or membrane-intact (green fluorescent), or dead or membrane-damaged (red fluorescent). Moribund sperm (combination of green and red fluorescence) were classified as membrane-damaged.

2.3. Semen processing

Immediately after collection, a standard semen evaluation was performed. Each ejaculate was then divided into aliquots as described below for each experiment. After adding pre-warmed semen extender, and immediately before centrifugation, sperm concentration, motility and membrane integrity were evaluated. Then, the aliquots were centrifuged as described below for

158 each experiment. Centrifugation duration included the time for rotor acceleration. An immediate
159 breaking feature was not used. The deceleration curve was the same for all treatments.
160 After centrifugation, 37 mL (40-mL suspensions) or 17 mL (20-mL suspensions) of the
161 supernatant was removed by aspiration with a 2-mL plastic transfer pipette. Transfer pipettes are
162 readily available and routinely used in andrology laboratories for aspiration of the supernatant.
163 Given the duration and forces used for centrifugation here, a tight pellet was obtained. The
164 supernatant was also removed immediately after centrifugation with minimal time delay.
165 Therefore, sperm loss in the supernatant due to swim up of spermatozoa was unlikely to occur.
166 Sperm concentration was evaluated in the supernatant with a hemacytometer [8,18] and semen
167 extender was added to re-suspend the pellet to a sperm concentration of 25 x 10^6 /mL. No
168 attempt was made to maintain the concentration of seminal plasma constant. Instead, semen was
169 processed using a routine protocol for cooling, where the final sperm concentration was taken
170 into account. Sperm motility and membrane integrity were assessed in the re-suspended semen
171 immediately. Re-suspended and non-centrifuged control samples were packaged in plastic bags
172 (Whirl-Pack, Nasco, Fort Atkinson, WI, USA), placed in a passive cooling device (Equitainer,
173 Hamilton Thorn Research, Danver, MA, USA) and stored at approximately 4 °C for 24 h. After
174 24 h of cold storage, semen was warmed at 37 °C for 10 min and sperm motility and membrane
175 integrity were reassessed.
176
177 2.4. Experiment 1: Effect of centrifugation force, duration and column height on sperm recovery
178 rate and survival
179

Each ejaculate (n = 7) was extended to a sperm concentration of 25 x 10^6 /mL with a milk-based semen extender (EZ-Mixin CST®, Animal Reproduction Systems). The extended semen was divided into nine aliquots. Each aliquot was centrifuged in a swinging bucket rotor centrifuge (Eppendorf 5804, Hamburg, Germany) at room temperature in a 50-mL conical tube under one of two centrifugation forces (400 or 900 x g), duration (5 or 10 min) and volumes (20 or 40 mL) (Table 1).

2.5. Experiment 2: Effect of sperm concentration on recovery rate and survival

Each ejaculate (n = 7) was divided into four aliquots and extended with a milk-based semen extender (EZ-Mixin CST®, Animal Reproduction Systems) to one of the following sperm concentrations: 1) 25 x 10^6/mL, uncentrifuged control; 2) 25 x 10^6/mL; 3) 50 x 10^6/mL; 4) 100 x 10^6/mL. Centrifugation of 40 mL of each aliquot was performed in a swinging bucket rotor centrifuge (Sorvall ST16, Fisher Scientific Co. LLC, Hanover Park, IL, USA) at room temperature in 50-mL conical tubes at 900 x g for 10 min. This centrifugal force and duration was chosen since it provided the best sperm yields in Exp. 1. After removing the supernatant, semen extender was added to dilute all aliquots to the same final sperm concentration of 25 x 10^6 /mL.

2.6. Experiment 3: Effect of centrifugation medium on sperm recovery rate and survival

Each ejaculate (n = 5) was divided into three aliquots. Each aliquot was diluted to a sperm concentration of 25 x 10^6/mL with a milk-based (EZ-Mixin CST®, Animal Reproduction

Systems), fractionated milk-based (INRA96, IMV Technologies, Maple Grove, MN, USA) or egg yolk-based (VMDZ, Partnar Animal Health, Port Huron, MI, USA) semen extender. Forty milliliters from each aliquot served as a non-centrifuged control sample. Other 40 mL from each aliquot were centrifuged in a swinging bucket rotor centrifuge (Sorvall ST16, Fisher Scientific Co. LLC) at room temperature in 50-mL conical tubes at 900 x g for 10 min. Since the goal of this experiment was to test the effect of centrifugation medium on sedimentation rates, all other centrifugation conditions were kept constant to eliminate any confounding effects of changing centrifugation conditions. After removing the supernatant, the corresponding semen extender was added to re-suspend the pellet to a final sperm concentration of 25 x 10^6 /mL.

2.7. Calculation of sperm yields and survival factors

Sperm yields after centrifugation were calculated as follows: Total sperm pre-centrifugation (TSP) (x 10^6) = initial sperm concentration x volume in the tube; Total sperm in the supernatant (TSS) (x 10^6) = sperm concentration in the supernatant x volume of the supernatant; Total sperm in the pellet (TSPe) (x 10^6) = TSP – TSS; Total yield (TY) = TSPe / TSP x 100; Total motile yield (TMY) = (TSPe x % total motility post-centrifugation) / (TSP x % total motility pre-centrifugation) x 100; Progressively motile yield (PMY) = (TSPe x % progressive motility post-centrifugation) / (TSP x % progressive motility pre-centrifugation) x 100; Viable yield (VY) = (TSPe x % viability post-centrifugation) / (TSP x % viability pre-centrifugation) x 100 [8,18].

Sperm motility and viability after centrifugation were normalized to the initial values, and the normalized variables were called survival factors [13,14]. Survival factor is more likely

226 to reveal differences between treatments since this variable eliminates the effect of individual
227 variation in initial semen quality on the outcome and assess only the changes in semen quality in
228 response to treatment [13,14]. Survival factors were calculated as follows: Survival factor for
229 total motility (SFT) = % total motility post-centrifugation / % total motility pre-centrifugation x
230 100; Survival factor for progressive motility (SFP) = % progressive motility post-centrifugation /
231 % progressive motility pre-centrifugation x 100; Survival factor for viability (SFV) = % viability
232 post-centrifugation / % viability pre-centrifugation x 100.
233 A similar normalization to values post-centrifugation was done after cooling: Survival
234 factor for total motility at 24 h (SFT24) = % total motility at 24 h / % total motility post-
235 centrifugation x 100; Survival factor for progressive motility at 24 h (SFP24) = % progressive
236 motility at 24 h / % progressive motility post-centrifugation x 100; Survival factor for viability at
237 24 h (SFV24) = % viability at 24 h / % viability post-centrifugation x 100.
238
239 2.8. Statistical analysis
240
241 Sperm yields and survival factors after centrifugation and cooling were tested for normality
242 using the Shapiro-Wilk test. Variables followed a normal distribution. The effect of
243 centrifugation protocol on the response variables (TY, TMY, PMY, VY, SFP, SFT, SFV, SFT24,
244 SFP24, SFV24) was evaluated with ANOVA for repeated measures within storage time
245 (immediately after centrifugation or after cooling). The general linear model procedure of SAS
246 package (SAS Institute, Cary, NC, USA) was used for analysis. The model included the random
247 effect of ejaculate and the fixed effect of treatment. In Exp. 1, each treatment represented a
248 different interaction of centrifugation force, duration and volume. In Exp. 2 and 3, each treatment

represented centrifugation with a different sperm concentration or semen extender, respectively. The control non-centrifuged treatments were also included in the models. If there was a significant treatment effect, pre-determined comparisons were made between treatments using least squares means with a Tukey adjustment of Type I error to 0.05. Differences were considered significant when $P < 0.05$. All values were expressed as mean ± SD.

3. Results

3.1. Experiment 1: Effect of centrifugation force, duration and column height on sperm recovery rate and survival

Initial total sperm motility was 78.1 ± 20.4 %, progressive sperm motility was 70.7 ± 22.4 % and sperm viability was 75.8 ± 14.9 %. There was a significant effect of ejaculate on all variables ($P < 0.05$) except TY, TMY and SFV24. After centrifugation, one stallion had a decrease in survival factors, one stallion had an improvement in semen quality, and five stallions had no apparent change.

Total sperm yield was greater for non-centrifuged semen (100 ± 0 %), semen centrifuged at 400 x g for 10 min in a 20-mL suspension (95.6 ± 5 %), 900 x g for 10 min in a 40-mL (91.4 ± 4.5 %) or 20-mL suspension (99.2 ± 0.8 %) and 900 x g for 5 min in a 20-mL suspension (93.8 ± 8.9 %) compared with semen centrifuged at 400 x g for 10 min in a 40-mL suspension (74.5 ± 7.6 %), 400 x g for 5 min in a 20-mL suspension (74.3 ± 8.6 %) and 900 x g for 5 min in a 40-mL suspension (72.6 ± 9.5 %), whereas centrifugation at 400 x g for 5 min in a 40-mL suspension provided the lowest total sperm yield (47.2 ± 7.3 %) ($P < 0.0001$). Total and

progressively motile sperm yields followed a similar pattern ($P < 0.0001$) (Table 1). Viable sperm yield was also highest for non-centrifuged semen (100 ± 0 %), semen centrifuged at 900 x g in 20-mL suspensions for 10 min (92 ± 18.5 %) or 5 min (87.8 ± 13.1 %), 400 x g for 10 min in a 20-mL suspension (86.9 ± 14.2 %) and 900 x g for 10 min in a 40-mL suspension (84.4 ± 19.3 %), and lowest after centrifugation at 400 x g for 5 min in a 40-mL suspension (44.5 ± 8 %) ($P < 0.0001$) (Table 1). Centrifugation protocol had no significant effect on any survival factor after centrifugation and cooling (Table 1).

3.2. Experiment 2: Effect of sperm concentration on recovery rate and survival

Initial total sperm motility was 76.6 ± 10.3 %, progressive sperm motility was 37.9 ± 40.4 % and sperm viability was 77.5 ± 16.8 %. None of the variables was affected by ejaculate, except SFT24 ($P = 0.0005$). While response to centrifugation at 25 and 50 x 10^6/mL was variable among stallions, all stallions had a decrease of ≥ 20 % in SFT24 h when semen was centrifuged at 100 x 10^6/mL. Neither TY nor TMY differed among centrifuged samples, however centrifugation at a sperm concentration of 100 x 10^6/mL resulted in significantly lower TY ($P = 0.0293$) and TMY ($P = 0.0219$) compared with non-centrifuged semen (Table 2). Viable yield was not different among centrifuged samples, however centrifugation at all concentrations resulted in significantly lower VY compared with non-centrifuged semen ($P = 0.0003$) (Table 2). Progressively motile yield was not different among treatments ($P = 0.0744$) (Table 2). None of the survival factors after centrifugation and cooling differed significantly among semen samples centrifuged at different concentrations, or compared with non-centrifuged semen (Table 2).

295 3.3. Experiment 3: Effect of centrifugation medium on sperm recovery rate and survival

296

297 Initial total sperm motility was 68.7 ± 13.4 %, progressive sperm motility was 36.6 ± 13.4 %
298 and sperm viability was 69.3 ± 24.9 %. There was no significant effect of ejaculate on any of the
299 variables, except SFT24 and SFP24 (P = 0.0005). Total sperm yield was not significantly
300 different among centrifuged samples, but centrifugation in INRA96 resulted in lower TY
301 compared to non-centrifuged semen (P =0.0022) (Table 3). Total and progressively motile, and
302 viable sperm yield were not significantly different among centrifuged samples. However,
303 centrifugation in VMDZ resulted in lower TMY (P = 0.0041), PMY (P = 0.0050) and VY (P =
304 0.0116) compared to non-centrifuged semen (Table 3). None of the survival factors after
305 centrifugation and cooling differed significantly among treatments, except SFP24. Semen
306 centrifuged in VMDZ had lower progressive motility after cooling compared with its non-
307 centrifuged control sample (P = 0.0344) (Table 3).

308

309 4. Discussion

310

311 The objectives of this study were to identify factors that affected sedimentation rates and
312 survival of equine spermatozoa after centrifugation. Possible delayed effects of centrifugation on
313 sperm function were assessed after 24 h of cold storage. The motile or viable yield in the pellet
314 and not the percent motility or viability is the parameter that best reflects the effectiveness of a
315 centrifugation protocol [13]. Also, because of the large variability in initial sperm motility and
316 viability among stallions, these parameters were normalized to eliminate this source of variation.
317 The normalized variables were called survival factors [13].

Survival factors after centrifugation were not affected by treatment in any of the experiments. Furthermore, no delayed effect of centrifugation on sperm motility and viability was evident after cooling for 24 h with most treatments. Only centrifugation in VMDZ resulted in a decrease in progressive motility after cooling. It can therefore be assumed that, under most of the conditions tested in this study, loss of motile or viable sperm was a result of a decrease in sedimentation rate through the supernatant rather than cell death or damage within the pellet.

The rate of sedimentation (v) of a particle is given by the following formula:

$$v = \frac{2r_p^2 (\rho_p - \rho_m) w^2 r}{9\eta (f/f_o)}$$

Where, r_p is the radius of the particle, ρ_p is the density of the particle, ρ_m is the density of the medium, w is the angular velocity of the rotor, r is the radial distance of the particle from the axis of rotation, η is the viscosity coefficient of the medium, f is the frictional coefficient of the hydrated aspherical particle, and f_o is the theoretical frictional coefficient of an unhydrated sphere of the same molecular mass and density [13,15]. Therefore, the rotational speed of the rotor, radial distance of the particles from the axis of rotation (given by the column height), and the density and viscosity of the medium affect sedimentation rate. While the radius, density and shape of the particle also affect sedimentation rate, these effects remain constant when comparing centrifugation protocols for a given cell type, such as sperm in the case of this study. As the centrifugal force increases, sedimentation rate also increases. The centrifugal force (G) is given by:

$$G = w^2 r$$

Hence, a particle experiences a greater force the further away it is from the axis of rotation. A shorter column height in a partially filled tube increases the minimum radial distance (Fig. 1).

341 Therefore, particles start to sediment at a higher gravitational field, have a reduced path length to
342 travel, and sedimentation is quicker [13-15].
343 In this study, 28 % of motile and viable sperm were lost with the supernatant after a
344 conventional centrifugation protocol at 400 x g for 10 min and a volume of 40 mL, which is
345 similar to other reports [7,8]. When the volume of the suspension was reduced to 20 mL,
346 resulting in a shorter column, sperm losses were significantly reduced to < 5 % after
347 centrifugation at a conventional force (400 x g) and duration (10 min). Total and viable sperm
348 yields were affected by the height of the suspension.
349 When centrifuging a conventional volume (40 mL) of semen in a 50-mL tube for a
350 conventional duration (10 min), increasing the centrifugal force to 900 x g also improved sperm
351 yields. Similar increases in sperm recovery rates after increasing centrifugal force were reported
352 previously [8,9,18]. Centrifugation duration and force are reciprocal, and total yield increases
353 linearly as the product of duration x force increases, until it plateaus at 100 %. The deleterious
354 effect of centrifugation on sperm function has been attributed to mechanical damage [14], tight
355 packing [14], and production of reactive oxygen species in the pellet [19]. Assuming cells are
356 damaged as a consequence of being packed within the pellet and not of sedimenting through the
357 supernatant, the viable and motile yields depend on the rate at which cells in the pellet are
358 damaged and the rate at which undamaged cells arrive in the pellet [13,14]. Once full
359 sedimentation is reached, viable and motile yields decrease as a consequence of cell damage in
360 the pellet and the lack of further arrival of undamaged cells [13,14]. Total yield almost reached
361 the plateau at 99 % when semen was centrifuged at 900 x g for 10 min in 20-mL suspensions.
362 Increasing the centrifugation duration or force beyond this seemed unnecessary when
363 centrifuging low volumes. Decreasing the centrifugation duration to 5 min resulted in decreased

364 sperm yields, except when semen was centrifuged in 20-mL suspensions at 900 x g. It seemed
365 then possible to decrease processing time using a higher force with small volumes of semen
366 without compromising recovery rates.
367 At any given centrifugation duration and force, sedimentation rate depends on the difference
368 in specific gravity between the cells and the surrounding medium, and the viscosity of the
369 medium [13-15]. Sedimentation rate increases as the density and viscosity of the medium
370 decrease [15]. Centrifugation medium affected recovery of sperm in this study. Density of the
371 media seemed similar among EZ mixin (1.0125 gr/mL), INRA96 (1.0095 gr/mL) and VMDZ
372 (1.011 gr/mL) semen extenders. However, it is possible that such a small difference in density
373 accounted for differences in sperm recovery. Viscosity of the media was not known and may
374 have been partly responsible for differences in sedimentation also. Centrifugation in INRA96
375 resulted in a significant loss of about 18 % of the initial sperm suspension compared with non-
376 centrifuged samples. However, survival factors for total and progressive motility were ≥ 100 %
377 since removing the supernatant and re-suspending the pellet in INRA96 resulted in an
378 improvement in sperm motility in four of the five stallions in this study. The ability of this semen
379 extender to improve sperm motility compensated for the lower sedimentation rate, and resulted
380 in no significant losses of motile sperm. These results cannot be extrapolated to conventional
381 centrifugation protocols. Total sperm yield after centrifugation in INRA96 at 400 x g for 10 min
382 was 54 % [9]. Using a higher centrifugation force may be recommended to minimize sperm
383 losses when using this semen extender.
384 On the other hand, VMDZ seemed unable to protect sperm from immediate and delayed
385 deleterious effects of centrifugation. A significant loss of total (31 %) and progressively motile
386 (13 %) sperm occurred after centrifugation in VMDZ compared to non-centrifuged semen.

Centrifugation in VMDZ resulted in an immediate reduction in sperm motility in four of the five stallions in the study. This may have accounted for the decrease in motile sperm yields in spite of the lack of difference in sedimentation rates. Furthermore, there was a dramatic 79 % decrease in progressive sperm motility after cooling semen centrifuged in VMDZ. Centrifugation in VMDZ resulted in a hard pellet that required prolonged pipetting for re-suspension. A loss of sperm motility and membrane integrity was reported after pipetting non-centrifuged rat and mouse sperm [20]. However, there seems to be a species difference in sensitivity of sperm to mechanical damage induced by pipetting since this procedure had no deleterious effects on bull, ram and boar sperm [20]. The effect of pipetting on equine sperm has not been critically evaluated and may have accounted for the immediate or delayed deleterious effects of centrifugation in VMDZ on sperm motility in this study. Also, removal of seminal plasma by centrifugation resulted in lower post-thaw sperm motility and higher lipid peroxidation when buck semen was frozen in an egg yolk-based extender compared with non-centrifuged semen, or centrifuged semen frozen in a soybean lecithin-based extender [21]. Seminal plasma is known to be a main source of antioxidant protection. It is therefore possible that the egg yolk-based semen extender was unable to provide sufficient antioxidant protection to support sperm progressive motility after centrifugation and cooling in the absence of seminal plasma.

The initial concentration of cell suspensions also influences sedimentation rate [15]. Density and viscosity of the medium may be influenced not only by the semen extender used but also by the amount of seminal plasma in the ejaculate, the ratio of semen: extender used or the sperm concentration in the suspension being centrifuged. In this study, sperm yield was affected by the concentration at which semen was centrifuged. Centrifugation at a high sperm concentration (100×10^6 /mL) resulted in significant sperm losses compared to non-centrifuged semen. It can

410 be speculated that this finding resulted from differences in density or viscosity of the medium
411 containing different concentrations of seminal plasma, or cell-to-cell interactions in the more
412 concentrated suspension. The properties of the pellet depend on the number of cells, which
413 determines the size of the pellet, centrifugal force and media composition [22]. An increase in
414 the number of cells results in a larger pellet. The larger the pellets the looser they are [22]. The
415 porosity and intermembrane distance between adjacent cells increase, likely due to repositioning
416 and changing orientation of the cells within a larger multi-layer pellet [22]. The larger pellet with
417 lower cell cohesion may have resulted in more cells aspirated with the supernatant rather than in
418 a decrease in sedimentation rate.

419 In conclusion, sperm survival after centrifugation and cooling was not affected by the
420 centrifugation protocol used. Only centrifugation in VMDZ resulted in a decrease in progressive
421 motility after centrifugation and cooling. When equine semen was centrifuged at 400 to 900 x g
422 for 5 to 10 min diluted to a sperm concentration of 25 to 100 x 10^6 /mL in milk- or fractionated
423 milk-based semen extenders, loss of motile or viable sperm resulted from a decrease in
424 sedimentation rate rather than cell death within the pellet. Therefore, centrifugation protocols
425 that improve sedimentation rate are likely to improve recovery of motile and viable sperm. With
426 conventional centrifugation protocols, centrifuging volumes of ≤ 20 mL in 50-mL tubes
427 minimized sperm losses in the supernatant. Due to the large volumes of semen that are often
428 processed, using a lower volume may not be practical in all circumstances. If 40-mL suspensions
429 are used, it may be recommended to increase the centrifugation force to 900 x g for 10 min.
430 When using this volume, force and duration, it may be recommended to centrifuge semen at a
431 sperm concentration of 25 to 50 x 10^6 /mL since centrifugation at a higher sperm concentration
432 resulted in significant sperm losses. Both milk- (EZ Mixin) and fractionated milk-based

(INRA96) semen extenders seemed equally suitable for centrifugation of equine semen under the conditions tested in this study. Use of an egg yolk-based semen extender (VMDZ) was not recommended for centrifugation due to a significant loss of motile spermatozoa and decrease in progressive sperm motility after cooling. Because there was an effect of stallion on some variables, the ideal centrifugation protocol may need to be adjusted for some individual stallions.

Disclosure statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1. Sperm yields and survival factors after centrifugation of equine semen at different forces (400 or 900 x g), duration (5 or 10 min) and volumes (20 or 40 mL), and after cooling for 24 h. TY = total yield, TMY = total motile yield, PMY = progressively motile yield, VY = viable yield, SFT = survival factor for total motility, SFP = survival factor for progressive motility, SFV = survival factor for viability, SFT24 = survival factor for total motility at 24 h, SFP24 = survival factor for progressive motility at 24 h, SFV24 = survival factor for viability at 24 h. ^{a,b,c,d,e}Within a row, values with different superscript differ significantly ($P < 0.0001$) (Mean ± SD).

Table 2. Sperm yields and survival factors after centrifugation of equine semen at 900 x g for 10 min in 50-mL tubes at different concentrations (25, 50 and 100 x 10^6/mL), and after cooling for 24 h. TY = total yield, TMY = total motile yield, PMY = progressively motile yield, VY = viable yield, SFT = survival factor for total motility, SFP = survival factor for progressive motility, SFV = survival factor for viability, SFT24 = survival factor for total motility at 24 h, SFP24 =

522 survival factor for progressive motility at 24 h, SFV24 = survival factor for viability at 24 h.

523 [a,b]Within a row, values with different superscript differ significantly ($P < 0.05$) (Mean ± SD).

524

525 Table 3. Sperm yields and survival factors after centrifugation at 900 x g for 10 min in 50-mL

526 tubes in different semen extenders (EZ mixin, INRA96 and VMDZ), and after cooling for 24 h.

527 TY = total yield, TMY = total motile yield, PMY = progressively motile yield, VY = viable

528 yield, SFT = survival factor for total motility, SFP = survival factor for progressive motility,

529 SFV = survival factor for viability, SFT24 = survival factor for total motility at 24 h, SFP24 =

530 survival factor for progressive motility at 24 h, SFV24 = survival factor for viability at 24 h.

531 [a,b]Within a row, values with different superscript differ significantly ($P < 0.05$) (Mean ± SD).

532

533 Fig.1. Simplified diagram of a swinging bucket rotor with the position of the tubes containing 40

534 mL (left) and 20 mL (right) of suspension during centrifugation. The centrifugal field is directed

535 radially outwards from the axis of rotation (arrowhead), and is given by the angular velocity of

536 the rotor and the radial distance of the particle from the axis of rotation. Even though the

537 maximum radial distance (distance to the bottom of the tube, r_{max}) is the same, the minimum

538 (distance to the meniscus, r_{min}) radial distance at the beginning of centrifugation is greater when

539 the tube is partially filled with 20 mL of suspension than with 40 mL.

	Centrifugation force, duration and volume								
Variable	0 x g 0 min 40 mL	400 x g 10 min 40 mL	400 x g 10 min 20 mL	400 x g 5 min 40 mL	400 x g 5 min 20 mL	900 x g 10 min 40 mL	900 x g 10 min 20 mL	900 x g 5 min 40 mL	900 x g 5 min 20 mL
TY (%)	100 ± 0^{a}	74.5 ± 7.6^{b}	95.6 ± 5^{a}	47.2 ± 7.3^{c}	74.3 ± 8.6^{b}	91.4 ± 4.5^{a}	99.2 ± 0.8^{a}	72.6 ± 9.5^{b}	93.8 ± 8.9^{a}
TMY (%)	100 ± 0^{a}	71.9 ± 13.3^{b}	97.2 ± 8.7^{a}	47.5 ± 10^{c}	71.2 ± 5.9^{b}	92.9 ± 9.1^{a}	96.2 ± 5.5^{a}	67.1 ± 10.6^{b}	94.9 ± 12^{a}
PMY (%)	100 ± 0^{a}	72.5 ± 15.2^{b}	100.4 ± 9.1^{a}	49.1 ± 11.2^{c}	74 ± 5.4^{b}	91.5 ± 11.3^{a}	99.5 ± 7.4^{a}	68.8 ± 12.9^{b}	95.7 ± 7.4^{a}
VY (%)	100 ± 0^{a}	$71.8 \pm 14^{b,c,d}$	$86.9 \pm 14.2^{a,b}$	44.5 ± 8^{e}	67.1 ± 7.6^{d}	$84.4 \pm 19.3^{a,b,c}$	92 ± 18.5^{a}	$69.2 \pm 11^{c,d}$	$87.8 \pm 13.1^{a,b}$
SFT (%)	100 ± 0	96.6 ± 15.3	101.7 ± 8.5	100.6 ± 15.9	96.4 ± 8.4	101.6 ± 8.2	96.9 ± 5.3	93.1 ± 13.6	101.2 ± 7.8
SFP (%)	100 ± 0	97.5 ± 18.4	105.1 ± 8.8	103.6 ± 16.7	100.1 ± 6.6	100.1 ± 10.4	100.3 ± 7.3	95.3 ± 15.9	102.5 ± 7.9
SFV (%)	100 ± 0	97.1 ± 19.6	91 ± 14.7	95.2 ± 15.1	91 ± 12.3	92 ± 18.5	92.8 ± 18.9	95.7 ± 12.4	94.1 ± 13.9
SFT24 (%)	88.5 ± 15	92.3 ± 12.3	92.6 ± 9.4	90.3 ± 9.4	85.2 ± 8.4	88.7 ± 12.1	87.2 ± 30.4	89.2 ± 19.1	90.7 ± 15.2
SFP24 (%)	86.5 ± 16.5	89.6 ± 14.5	91.5 ± 15.6	82.3 ± 10.9	84.2 ± 11.1	89.1 ± 10.6	86.8 ± 35	80.9 ± 13.9	89.8 ± 16.7
SFV24 (%)	96.4 ± 5.4	94.7 ± 14.9	103.4 ± 17.2	92.5 ± 10.1	97.3 ± 11.3	87.1 ± 11.7	100.9 ± 15.9	96.3 ± 10	100 ± 11.1

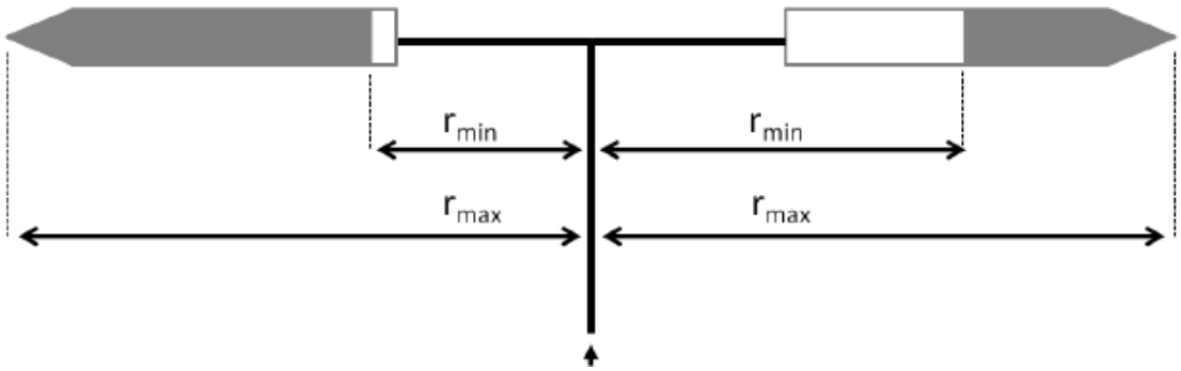
	Sperm concentration (x 10^6 /mL)			
Variable	Control	25	50	100
TY (%)	100 ± 0^{a}	$91.3 \pm 6.4^{a,b}$	$85.4 \pm 15.7^{a,b}$	83.8 ± 10.7^{b}
TMY (%)	100 ± 0^{a}	$81.6 \pm 12.5^{a,b}$	$83.8 \pm 20.7^{a,b}$	81.7 ± 6.8^{b}
PMY (%)	100 ± 0	80.6 ± 27.8	73.9 ± 22	76.4 ± 23.5
VY (%)	100 ± 0^{a}	70.6 ± 3.9^{b}	65.5 ± 17.7^{b}	67.1 ± 14^{b}
SFT (%)	100 ± 0	90.3 ± 6.6	97.5 ± 7.7	93.1 ± 30.8
SFP (%)	100 ± 0	88.3 ± 19.9	89.3 ± 22.6	93.1 ± 30.8
SFV (%)	100 ± 0	83.9 ± 13.9	82.5 ± 18.9	82.7 ± 17.7
SFT24 (%)	79.2 ± 13.9	74.8 ± 21.1	69.1 ± 19.5	62.3 ± 20.7
SFP24 (%)	58.1 ± 33.2	53.1 ± 36.3	38.1 ± 18.4	46.7 ± 60.4
SFV24 (%)	84.9 ± 18.7	98.2 ± 26.4	91.5 ± 9.1	105.9 ± 30.2

542

543

	Semen extender					
Variable	INRA96 Control	INRA96 Centrifuged	VMDZ Control	VMDZ Centrifuged	EZ Mixin Control	EZ Mixin Centrifuged
TY (%)	100 ± 0[a]	81.8 ± 11.3[b]	100 ± 0[a]	86.7 ± 17. 4[a,b]	100 ± 0[a]	93.5 ± 2.7[a,b]
TMY (%)	100 ± 0[a]	81.5 ± 14.9[a,b]	100 ± 0[a]	69.3 ± 22.6[b]	100 ± 0[a]	83.7 ± 18.4[a,b]
PMY (%)	100 ± 0[a]	86.6 ± 27.2[a,b]	100 ± 0 [a]	63.5 ± 18.2[b]	100 ± 0[a]	89.9 ± 10.1[a,b]
VY (%)	100 ± 0[a]	68.4 ± 30.6[a,b]	100 ± 0[a]	60.9 ± 36.5[b]	100 ± 0[a]	81.4 ± 15.3[a,b]
SFT (%)	100 ± 0	99.6 ± 11.1	100 ± 0	85 ± 19.1	100 ± 0	90 ± 17
SFP (%)	100 ± 0	104.9 ± 23.1	100 ± 0	80.9 ± 20.9	100 ± 0	93.1 ± 13.5
SFV (%)	100 ± 0	82.4 ± 31.7	100 ± 0	76.1 ± 33.7	100 ± 0	88.6 ± 13.1
SFT24 (%)	70.7 ± 18.9	63 ± 11.2	71.1 ± 41.6	44.3 ± 6.6	69.3 ± 34.1	54.4 ± 25.9
SFP24 (%)	64.5 ± 31.7[a,b]	36.9 ± 14.2[a,b]	70.8 ± 56.1[a]	21 ± 10.8[b]	48 ± 46.7[a,b]	36.9 ± 30.7[a,b]
SFV24 (%)	86.4 ± 13	106.4 ± 41.8	114.3 ± 48.5	98.8 ± 37.5	62.8 ± 32.1	85.4 ± 33.4

549 Figure 1.



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