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


How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:


Published Version Information


Copyright: © 2012 Elsevier Inc.


Publisher’s Link: http://www.theriojournal.com/article/S0093-691X%2812%2900411-6/abstract

This item was retrieved from the K-State Research Exchange (K-REx), the institutional repository of Kansas State University. K-REx is available at http://krex.ksu.edu
Factors affecting sperm recovery rates and survival after centrifugation of equine semen

M.S. Ferrera*, S.K. Lylea, B.E. Eiltsa, A. H. Eljarrah¹, D.L. Paccamontia

aEquine Health Studies Program, Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA 70803

* Corresponding author and present address at: Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA. Tel.: +1-785-532-5700. Fax: +1-785-532-4989. Email address: mferrer@vet.k-state.edu

¹Present address: Department of Veterinary Clinical Sciences, Jordan University of Science and Technology, Irbid, Jordan
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.
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
each experiment. Centrifugation duration included the time for rotor acceleration. An immediate breaking feature was not used. The deceleration curve was the same for all treatments.

After centrifugation, 37 mL (40-mL suspensions) or 17 mL (20-mL suspensions) of the supernatant was removed by aspiration with a 2-mL plastic transfer pipette. Transfer pipettes are readily available and routinely used in andrology laboratories for aspiration of the supernatant.

Given the duration and forces used for centrifugation here, a tight pellet was obtained. The supernatant was also removed immediately after centrifugation with minimal time delay. Therefore, sperm loss in the supernatant due to swim up of spermatozoa was unlikely to occur.

Sperm concentration was evaluated in the supernatant with a hemacytometer [8,18] and semen extender was added to re-suspend the pellet to a sperm concentration of $25 \times 10^6$ /mL. No attempt was made to maintain the concentration of seminal plasma constant. Instead, semen was processed using a routine protocol for cooling, where the final sperm concentration was taken into account. Sperm motility and membrane integrity were assessed in the re-suspended semen immediately. Re-suspended and non-centrifuged control samples were packaged in plastic bags (Whirl-Pack, Nasco, Fort Atkinson, WI, USA), placed in a passive cooling device (EQUITAINER, Hamilton Thorn Research, Danver, MA, USA) and stored at approximately 4 °C for 24 h. After 24 h of cold storage, semen was warmed at 37 °C for 10 min and sperm motility and membrane integrity were reassessed.

2.4. Experiment 1: Effect of centrifugation force, duration and column height on sperm recovery rate and survival
Each ejaculate (n = 7) was extended to a sperm concentration of $25 \times 10^6$/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) $25 \times 10^6$/mL, uncentrifuged control; 2) $25 \times 10^6$/mL; 3) $50 \times 10^6$/mL; 4) $100 \times 10^6$/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 $25 \times 10^6$/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 $25 \times 10^6$/mL with a milk-based (EZ-Mixin CST®, Animal Reproduction Systems) 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).
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
to reveal differences between treatments since this variable eliminates the effect of individual variation in initial semen quality on the outcome and assess only the changes in semen quality in response to treatment [13,14]. Survival factors were calculated as follows: Survival factor for total motility (SFT) = % total motility post-centrifugation / % total motility pre-centrifugation x 100; Survival factor for progressive motility (SFP) = % progressive motility post-centrifugation / % progressive motility pre-centrifugation x 100; Survival factor for viability (SFV) = % viability post-centrifugation / % viability pre-centrifugation x 100.

A similar normalization to values post-centrifugation was done after cooling: Survival factor for total motility at 24 h (SFT24) = % total motility at 24 h / % total motility post-centrifugation x 100; Survival factor for progressive motility at 24 h (SFP24) = % progressive motility at 24 h / % progressive motility post-centrifugation x 100; Survival factor for viability at 24 h (SFV24) = % viability at 24 h / % viability post-centrifugation x 100.

2.8. Statistical analysis

Sperm yields and survival factors after centrifugation and cooling were tested for normality using the Shapiro-Wilk test. Variables followed a normal distribution. The effect of centrifugation protocol on the response variables (TY, TMY, PMY, VY, SFP, SFT, SFV, SFT24, SFP24, SFV24) was evaluated with ANOVA for repeated measures within storage time (immediately after centrifugation or after cooling). The general linear model procedure of SAS package (SAS Institute, Cary, NC, USA) was used for analysis. The model included the random effect of ejaculate and the fixed effect of treatment. In Exp. 1, each treatment represented a 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 10⁶/mL was variable among stallions, all stallions had a decrease of ≥ 20 % in SFT24 h when semen was centrifuged at 100 x 10⁶/mL. Neither TY nor TMY differed among centrifuged samples, however centrifugation at a sperm concentration of 100 x 10⁶/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).
3.3. Experiment 3: Effect of centrifugation medium on sperm recovery rate and survival

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

4. Discussion

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

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

Where, \( r_p \) is the radius of the particle, \( \rho_p \) is the density of the particle, \( \rho_m \) 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, \( \eta \) 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).
Therefore, particles start to sediment at a higher gravitational field, have a reduced path length to travel, and sedimentation is quicker [13-15].

In this study, 28 % of motile and viable sperm were lost with the supernatant after a conventional centrifugation protocol at 400 x g for 10 min and a volume of 40 mL, which is similar to other reports [7,8]. When the volume of the suspension was reduced to 20 mL, resulting in a shorter column, sperm losses were significantly reduced to < 5 % after centrifugation at a conventional force (400 x g) and duration (10 min). Total and viable sperm yields were affected by the height of the suspension.

When centrifuging a conventional volume (40 mL) of semen in a 50-mL tube for a conventional duration (10 min), increasing the centrifugal force to 900 x g also improved sperm yields. Similar increases in sperm recovery rates after increasing centrifugal force were reported previously [8,9,18]. Centrifugation duration and force are reciprocal, and total yield increases linearly as the product of duration x force increases, until it plateaus at 100 %. The deleterious effect of centrifugation on sperm function has been attributed to mechanical damage [14], tight packing [14], and production of reactive oxygen species in the pellet [19]. Assuming cells are damaged as a consequence of being packed within the pellet and not of sedimenting through the supernatant, the viable and motile yields depend on the rate at which cells in the pellet are damaged and the rate at which undamaged cells arrive in the pellet [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]. Total yield almost reached the plateau at 99 % when semen was centrifuged at 900 x g for 10 min in 20-mL suspensions. Increasing the centrifugation duration or force beyond this seemed unnecessary when centrifuging low volumes. Decreasing the centrifugation duration to 5 min resulted in decreased
sperm yields, except when semen was centrifuged in 20-mL suspensions at 900 x g. It seemed then possible to decrease processing time using a higher force with small volumes of semen without compromising recovery rates.

At any given centrifugation duration and force, sedimentation rate depends on the difference in specific gravity between the cells and the surrounding medium, and the viscosity of the medium [13-15]. Sedimentation rate increases as the density and viscosity of the medium decrease [15]. Centrifugation medium affected recovery of sperm in this study. Density of the media seemed similar among EZMixin (1.0125 gr/mL), INRA96 (1.0095 gr/mL) and VMDZ (1.011 gr/mL) semen extenders. However, it is possible that such a small difference in density accounted for differences in sperm recovery. Viscosity of the media was not known and may have been partly responsible for differences in sedimentation also. Centrifugation in INRA96 resulted in a significant loss of about 18 % of the initial sperm suspension compared with non-centrifuged samples. However, survival factors for total and progressive motility were \( \geq 100 \% \) since removing the supernatant and re-suspending the pellet in INRA96 resulted in an improvement in sperm motility in four of the five stallions in this study. The ability of this semen extender to improve sperm motility compensated for the lower sedimentation rate, and resulted in no significant losses of motile sperm. These results cannot be extrapolated to conventional centrifugation protocols. Total sperm yield after centrifugation in INRA96 at 400 x g for 10 min was 54 % [9]. Using a higher centrifugation force may be recommended to minimize sperm losses when using this semen extender.

On the other hand, VMDZ seemed unable to protect sperm from immediate and delayed deleterious effects of centrifugation. A significant loss of total (31 %) and progressively motile (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 x 10^6/mL) resulted in significant sperm losses compared to non-centrifuged semen. It can
be speculated that this finding resulted from differences in density or viscosity of the medium containing different concentrations of seminal plasma, or cell-to-cell interactions in the more concentrated suspension. The properties of the pellet depend on the number of cells, which determines the size of the pellet, centrifugal force and media composition [22]. An increase in the number of cells results in a larger pellet. The larger the pellets the looser they are [22]. The porosity and intermembrane distance between adjacent cells increase, likely due to repositioning and changing orientation of the cells within a larger multi-layer pellet [22]. The larger pellet with lower cell cohesion may have resulted in more cells aspirated with the supernatant rather than in a decrease in sedimentation rate.

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

Acknowledgements

The authors thank Joanna Kouba and Bradley Purdue from the Department of Animal Sciences, Kansas State University for providing semen samples. Thanks to Dr. Robert Larson from the Department of Clinical Sciences, Kansas State University for his guidance with the statistical analysis.

References


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 10⁶/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.

\[ a,b \text{Within a row, values with different superscript differ significantly (P < 0.05) (Mean ± SD).} \]

Table 3. Sperm yields and survival factors after centrifugation at 900 x g for 10 min in 50-mL tubes in different semen extenders (EZ mixin, INRA96 and VMDZ), 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.

\[ a,b \text{Within a row, values with different superscript differ significantly (P < 0.05) (Mean ± SD).} \]

Fig.1. Simplified diagram of a swinging bucket rotor with the position of the tubes containing 40 mL (left) and 20 mL (right) of suspension during centrifugation. The centrifugal field is directed radially outwards from the axis of rotation (arrowhead), and is given by the angular velocity of the rotor and the radial distance of the particle from the axis of rotation. Even though the maximum radial distance (distance to the bottom of the tube, \( r_{\text{max}} \)) is the same, the minimum (distance to the meniscus, \( r_{\text{min}} \)) radial distance at the beginning of centrifugation is greater when the tube is partially filled with 20 mL of suspension than with 40 mL.
<table>
<thead>
<tr>
<th>Variable</th>
<th>0 x g</th>
<th>400 x g</th>
<th>400 x g</th>
<th>400 x g</th>
<th>400 x g</th>
<th>900 x g</th>
<th>900 x g</th>
<th>900 x g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>40 mL</td>
<td>10 min</td>
<td>10 min</td>
<td>5 min</td>
<td>5 min</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>40 mL</td>
<td>20 mL</td>
<td>40 mL</td>
<td>20 mL</td>
<td>40 mL</td>
<td>20 mL</td>
<td>40 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>TY (%)</td>
<td>100 ± 0a</td>
<td>74.5 ± 7.6b</td>
<td>95.6 ± 5a</td>
<td>47.2 ± 7.3c</td>
<td>74.3 ± 8.6b</td>
<td>91.4 ± 4.5a</td>
<td>99.2 ± 0.8a</td>
<td>72.6 ± 9.5b</td>
</tr>
<tr>
<td>TMY (%)</td>
<td>100 ± 0a</td>
<td>71.9 ± 13.3b</td>
<td>97.2 ± 8.7a</td>
<td>47.5 ± 10c</td>
<td>71.2 ± 5.9b</td>
<td>92.9 ± 9.1a</td>
<td>96.2 ± 5.5a</td>
<td>67.1 ± 10.6b</td>
</tr>
<tr>
<td>PMY (%)</td>
<td>100 ± 0a</td>
<td>72.5 ± 15.2b</td>
<td>100.4 ± 9.1a</td>
<td>49.1 ± 11.2c</td>
<td>74 ± 5.4b</td>
<td>91.5 ± 11.3a</td>
<td>99.5 ± 7.4a</td>
<td>68.8 ± 12.9b</td>
</tr>
<tr>
<td>VY (%)</td>
<td>100 ± 0a</td>
<td>71.8 ± 14b,cd</td>
<td>86.9 ± 14.2ab,</td>
<td>44.5 ± 8e</td>
<td>67.1 ± 7.6d</td>
<td>84.4 ± 19.3ab,cd</td>
<td>92 ± 18.5a</td>
<td>69.2 ± 11cd</td>
</tr>
<tr>
<td>SFT (%)</td>
<td>100 ± 0</td>
<td>96.6 ± 15.3</td>
<td>101.7 ± 8.5</td>
<td>100.6 ± 15.9</td>
<td>96.4 ± 8.4</td>
<td>101.6 ± 8.2</td>
<td>96.9 ± 5.3</td>
<td>93.1 ± 13.6</td>
</tr>
<tr>
<td>SFP (%)</td>
<td>100 ± 0</td>
<td>97.5 ± 18.4</td>
<td>105.1 ± 8.8</td>
<td>103.6 ± 16.7</td>
<td>100.1 ± 6.6</td>
<td>100.1 ± 10.4</td>
<td>100.3 ± 7.3</td>
<td>95.3 ± 15.9</td>
</tr>
<tr>
<td>SFV (%)</td>
<td>100 ± 0</td>
<td>97.1 ± 19.6</td>
<td>91 ± 14.7</td>
<td>95.2 ± 15.1</td>
<td>91 ± 12.3</td>
<td>92 ± 18.5</td>
<td>92.8 ± 18.9</td>
<td>95.7 ± 12.4</td>
</tr>
<tr>
<td>SFT24 (%)</td>
<td>88.5 ± 15</td>
<td>92.3 ± 12.3</td>
<td>92.6 ± 9.4</td>
<td>90.3 ± 9.4</td>
<td>85.2 ± 8.4</td>
<td>88.7 ± 12.1</td>
<td>87.2 ± 30.4</td>
<td>89.2 ± 19.1</td>
</tr>
<tr>
<td>SFP24 (%)</td>
<td>86.5 ± 16.5</td>
<td>89.6 ± 14.5</td>
<td>91.5 ± 15.6</td>
<td>82.3 ± 10.9</td>
<td>84.2 ± 11.1</td>
<td>89.1 ± 10.6</td>
<td>86.8 ± 35</td>
<td>80.9 ± 13.9</td>
</tr>
<tr>
<td>SFV24 (%)</td>
<td>96.4 ± 5.4</td>
<td>94.7 ± 14.9</td>
<td>103.4 ± 17.2</td>
<td>92.5 ± 10.1</td>
<td>97.3 ± 11.3</td>
<td>87.1 ± 11.7</td>
<td>100.9 ± 15.9</td>
<td>96.3 ± 10</td>
</tr>
</tbody>
</table>

Centrifugation force, duration and volume
<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>TY (%)</td>
<td>100 ± 0(^a)</td>
<td>91.3 ± 6.4(^{a,b})</td>
<td>85.4 ± 15.7(^{a,b})</td>
<td>83.8 ± 10.7(^b)</td>
</tr>
<tr>
<td>TMY (%)</td>
<td>100 ± 0(^a)</td>
<td>81.6 ± 12.5(^{a,b})</td>
<td>83.8 ± 20.7(^{a,b})</td>
<td>81.7 ± 6.8(^b)</td>
</tr>
<tr>
<td>PMY (%)</td>
<td>100 ± 0</td>
<td>80.6 ± 27.8</td>
<td>73.9 ± 22</td>
<td>76.4 ± 23.5</td>
</tr>
<tr>
<td>VY (%)</td>
<td>100 ± 0(^a)</td>
<td>70.6 ± 3.9(^b)</td>
<td>65.5 ± 17.7(^b)</td>
<td>67.1 ± 14(^b)</td>
</tr>
<tr>
<td>SFT (%)</td>
<td>100 ± 0</td>
<td>90.3 ± 6.6</td>
<td>97.5 ± 7.7</td>
<td>93.1 ± 30.8</td>
</tr>
<tr>
<td>SFP (%)</td>
<td>100 ± 0</td>
<td>88.3 ± 19.9</td>
<td>89.3 ± 22.6</td>
<td>93.1 ± 30.8</td>
</tr>
<tr>
<td>SFV (%)</td>
<td>100 ± 0</td>
<td>83.9 ± 13.9</td>
<td>82.5 ± 18.9</td>
<td>82.7 ± 17.7</td>
</tr>
<tr>
<td>SFT24 (%)</td>
<td>79.2 ± 13.9</td>
<td>74.8 ± 21.1</td>
<td>69.1 ± 19.5</td>
<td>62.3 ± 20.7</td>
</tr>
<tr>
<td>SFP24 (%)</td>
<td>58.1 ± 33.2</td>
<td>53.1 ± 36.3</td>
<td>38.1 ± 18.4</td>
<td>46.7 ± 60.4</td>
</tr>
<tr>
<td>SFV24 (%)</td>
<td>84.9 ± 18.7</td>
<td>98.2 ± 26.4</td>
<td>91.5 ± 9.1</td>
<td>105.9 ± 30.2</td>
</tr>
<tr>
<td>Variable</td>
<td>INRA96 Control</td>
<td>INRA96 Centrifuged</td>
<td>VMDZ Control</td>
<td>VMDZ Centrifuged</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>TY (%)</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.8 ± 11.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.7 ± 17.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMY (%)</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.5 ± 14.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.3 ± 22.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PMY (%)</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.6 ± 27.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.5 ± 18.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VY (%)</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.4 ± 30.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.9 ± 36.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFT (%)</td>
<td>100 ± 0</td>
<td>99.6 ± 11.1</td>
<td>100 ± 0</td>
<td>85 ± 19.1</td>
</tr>
<tr>
<td>SFP (%)</td>
<td>100 ± 0</td>
<td>104.9 ± 23.1</td>
<td>100 ± 0</td>
<td>80.9 ± 20.9</td>
</tr>
<tr>
<td>SFV (%)</td>
<td>100 ± 0</td>
<td>82.4 ± 31.7</td>
<td>100 ± 0</td>
<td>76.1 ± 33.7</td>
</tr>
<tr>
<td>SFT24 (%)</td>
<td>70.7 ± 18.9</td>
<td>63 ± 11.2</td>
<td>71.1 ± 41.6</td>
<td>44.3 ± 6.6</td>
</tr>
<tr>
<td>SFP24 (%)</td>
<td>64.5 ± 31.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.9 ± 14.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>70.8 ± 56.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 ± 10.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFV24 (%)</td>
<td>86.4 ± 13</td>
<td>106.4 ± 41.8</td>
<td>114.3 ± 48.5</td>
<td>98.8 ± 37.5</td>
</tr>
</tbody>
</table>
Figure 1.