Research Report

Pipette or vortex are equally effective for mixing stallion sperm after cushion centrifugation

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Abstract

Data on effects of mixing technique after cushion centrifugation on stallion sperm quality and longevity are limited. Objective was to compare mixing sperm via pipette or vortex after centrifugation. In Experiment 1, effects of 2 vortex speeds (minimum and maximum) and 3 vortex intervals (5, 15, and 30 seconds), and mixing (20 and 100 times) with a 1,000 µl pipette on sperm quality and sperm clumping were determined. There was no effect ($p > 0.05$) of treatment on total motility, progressive motility, curvilinear velocity, number of detached heads, or size of sperm clumps. There was an effect ($p = 0.05$) of treatment on number of sperm clumps, with fewer clumps in vortexed sample. In Experiment 2, effects of minimum vortex speed for 15 seconds, maximum vortex speed for 15 seconds and pipetting 20 times with a 1,000 µl pipette on sperm parameters and sperm clumping and at 24 and 48 hours of cooled storage were determined. There was an effect ($p = 0.0001$) of treatment on sperm viability (higher immediately after collection) but no difference among treatments. There was no effect ($p > 0.05$) of treatment on total motility, progressive motility, curvilinear velocity, number of sperm clumps, nor size of sperm clumps at 24 or 48 hours. We concluded that either a vortex or pipette can be used to mix sperm after cushion centrifugation with no detrimental effect on sperm quality or longevity.

Keywords: Stallion, sperm, centrifugation, mixing, vortex, pipette

Introduction

Cushion centrifugation is a useful technique to concentrate stallion semen and is routinely performed.[1] Generally, after cushion centrifugation the supernatant and cushion media are removed, and semen is reconstituted with extender. Removal of cushion is optional as cushion in reextended sperm had no effect on sperm parameters and fertility.[2] Most laboratories resuspend sperm pellet in extender by mixing with a pipette; however, some use a vortex (Schnobrich M., unpublished observation). There are limited data on the impact of various mixing methods on sperm quality after centrifugation.

Agitation methods are commonly used in biopharmaceutical industry for anything from mixing to mass transfer.[3] Various agitation methods can be used; however, they introduce mechanical stresses to container and sample. Vortex mixers are common in laboratories and feature a rotary motor with an offset cupped rubber piece. When a tube is pressed into the cupped rubber piece, a motor is activated and because of offset rubber piece a swirling motion occurs that mixes the suspension. This introduces shear stresses into the system[3] and mixing layers can potentially form at different velocities, resulting in turbulent flow.[4] These factors could improve mixing but also could have a deleterious effect on sperm. Objective was to compare mixing sperm via pipette or vortex after centrifugation. Our hypothesis was that vortex reduces sperm clumps and increases tailless heads compared to pipette.

Materials and methods

Animal use was approved by the Institutional Animal Care and Use Committee (IACUC 21-04-STW).

Experiment 1

A pilot study was conducted to determine effects of various vortex speeds and duration on sperm and sperm clumping.

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Citation: Clinical Theriogenology 2024, 16, 10782, http://dx.doi.org/10.58292/CT.v16.10782

Three ejaculates from 1 stallion were utilized; stallion was collected using a Missouri artificial vagina, phantom, and tease mare. After the gel-free semen was collected the volume of semen, concentration (NucleoCounter®, ChemoMetec A/S, Allerod, Denmark), total motility, progressive motility, curvilinear velocity (VCL; IVOSii, Hamilton Thorne, Beverly, MA, USA) were determined.[5] A sample from the initial ejaculate was diluted with 10% buffered formalin saline (BFS; Formalin 10, Animal Reproduction Systems, Chino, CA, USA) to evaluate sperm morphology.[6] Semen was then extended 1:1 (INRA96; IMV Technologies, Osseo, MN, USA) and 10 ml was aliquoted to 8 conical tubes (15 ml; Falcon™, Fisher Scientific, Waltham, MA, USA). Then, 1 ml of cushion centrifugation media (Red Cushion, Botupharma USA, Phoenix, AZ, USA) was layered underneath the semen-extender mix using a 3 ml syringe with a Tom Cat catheter (Argyle™ open end catheter with 14 cm adaptor, Covidien, Dublin, Ireland) attached. Conical tubes were balanced and centrifuged at 1,000 x g for 20 minutes. After centrifugation, a vacuum aspirator was used to remove the supernatant and a syringe with Tom Cat catheter was used to remove the cushion media. Semen was resuspended with extender (INRA96) to a concentration of ~ 25 x 10^6 sperm/ml and evaluated with a cell counter (NucleoCounter®). Conical tubes were allocated to the following groups to mix the resuspended sperm-extender solution: vortex minimum speed 5 seconds (Min5s); vortex minimum speed 15 seconds (Min15s); vortex minimum speed 30 seconds (Min30s); vortex maximum speed 5 seconds (Max5s); vortex maximum speed 15 seconds (Max15s); vortex maximum speed 30 seconds (Max30s); mixing via 1,000 µl pipette (Eppendorf North America, Enfield, CT, USA) 20 times (P20); and mixing via 1,000 µl pipette 100 times (P100). Motility parameters were assessed after centrifugation and mixing, and a sample from each treatment was diluted with 10% buffered formalin saline to evaluate sperm morphology. For sperm clumping, samples were diluted 1:1 with 10% BFS.

Sperm morphological evaluation was performed using DIC microscopy at 1,250 x magnification. Sperm were classified as normal, abnormal heads, abnormal acrosomes, tailless heads, distal cytoplasmic droplets, proximal cytoplasmic droplets, abnormal midpieces, bent midpieces, bent tails, coiled tails, and premature germ cells.[7] Only the number of normal sperm and tailless heads were used for analysis.

Clumping was defined as 3 or more sperm aggregated together. A Makler® counting chamber (Sefi-Medical Instruments, LTD, Santa Ana, CA, USA) was utilized for counting clumps. After semen mixture was well mixed, a 1.5 µl drop was placed on the center of the disc area using a micropipette. A coverslip was then placed on the 4 pins and gently pressed down. Sperm clumps were counted within each grid and those that touched the top or left lines, whereas those touching the bottom or right lines were not counted. A line of 10 squares were counted and multiplied by 2 (to account for dilution), this represented the sperm clump concentration (10^6/ml). A second strip of 10 squares was counted and sperm clump concentration was determined as before; an average of 2 counts was used to determine sperm clump concentration. Each time a sperm clump was observed the number of sperm in the clump was counted and recorded.

Experiment 2

Seven stallions were each collected between 1 to 3 times to produce a total of 12 collections. Semen was initially analyzed as described above. A sample from the initial ejaculate was diluted with 10% buffered formalin saline (BFS) to evaluate sperm morphology. Semen was then extended 1:1 (INRA96) and aliquoted to 3 conical tubes (50 ml; Falcon™, Fisher Scientific) and 1 ml of cushion centrifugation media (Red Cushion) was layered underneath the semen-extender mix using a 3 ml syringe with a Tom Cat catheter attached. Conical tubes were balanced and centrifuged at 1,000 x g for 20 minutes. After centrifugation, a vacuum aspirator was used to remove the supernatant and a syringe with Tom Cat catheter was used to remove most of the cushion media. Sperm pellet was resuspended with extender (INRA96) to a concentration of ~ 200 x 10^6 sperm/ml and evaluated with a cell counter (NucleoCounter®). Conical tubes were allocated to the following groups to mix: minimum vortex speed for 15 seconds, maximum vortex speed for 15 seconds and pipetting 20 times (1,000 µl pipette). A sample from each group was then stored in a passive cooling system (EquitainerÒ) for 48 hours. Sperm concentration, viability and motility parameters were assessed at 24 hours and 48 hours of cooling after centrifugation. A sample from each treatment was taken at 24 and 48 hours and diluted with 10% BFS to evaluate sperm morphology. Undiluted samples from 24 and 48 hours were evaluated for sperm clumping. Sperm morphology was evaluated as described above.

Counting the number of sperm clumps was performed as described above, except for multiplication by 2, as the samples were not diluted in FBS as performed in Experiment 1. Additionally, clump sizes were categorized based on the number of sperm per clump: 3-5 sperm (1), 5-10 sperm (2), and > 10 sperm (3).

Data analyses

For Experiment 1, total motility, progressive motility, curvilinear velocity (VCL), number of sperm clumps, size of sperm clumps, sperm with normal morphology, and sperm with detached heads were analyzed using SAS (9.4) Mixed procedure with treatment and collection as fixed effects, and treatment and collection interaction as random effects. Pairwise comparisons were made between treatments using PDIFF option. Data are expressed as LSmeans ± SEM.

For Experiment 2, sperm viability, total motility, progressive motility, curvilinear velocity (VCL), straightness, number of sperm clumps, sperm with normal morphology, and sperm with detached heads were analyzed using SAS (9.4) General Linear Model procedure with collection, stallion, treatment and time as fixed effects and interaction between time and treatment, stallion and treatment, stallion and collection, and stallion and time as random effects. Means were determined and when there were interaction ($p < 0.05$) comparisons were made using a Tukey test. Clump size was analyzed using SAS (9.4) Logistic Regression with collection, stallion, treatment and time as fixed effects and interaction between time and treatment, stallion and treatment, stallion and collection, and stallion and time as random effects. The proportion clump sizes by treatment and time by clump size by treatment were determined using the Frequency procedure with a Chi square procedure invoked. Significance was set at $p \leq 0.05$.

Results

In Experiment 1, there was no effect of treatment on total motility ($p = 0.97$), progressive motility ($p = 0.62$), VCL

Citation: Clinical Theriogenology 2024, 16, 10782, http://dx.doi.org/10.58292/CT.v16.10782

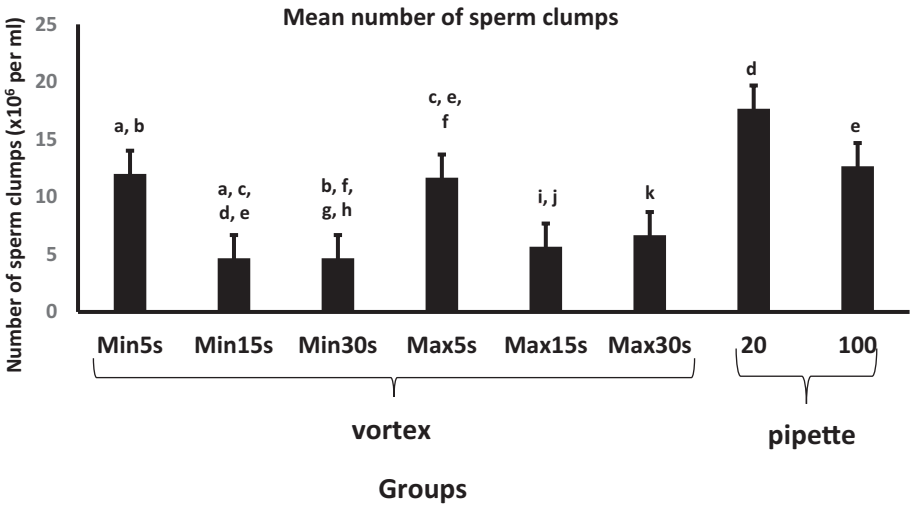

Figure 1. Mean number of sperm clumps in centrifuged samples after vortexing at minimum (Min) speed for 5, 15 or 30 seconds; at maximum speed for 5, 15 or 30 seconds; and after mixing with pipette for 20 or 100 aspirations. Groups without common superscripts differed ($p < 0.05$).

($p = 0.16$), number of detached heads ($p = 0.27$) or size of sperm clumps ($p = 0.16$). There was an effect of treatment on the number of sperm clumps ($p = 0.05$; Figure 1). The Min15s and Min30s treatments had fewest clumps (4.7 ± 2.0 and 4.7 ± 2.0, respectively), followed by Max15s (5.7 ± 2.0), Max30s (6.7 ± 2.0), Max5s (11.7 ± 2.0), Min5s (12.0 ±2.0), P100 (12.7 ± 2.0), and P20 (17.7 ± 2.0; Figure 1).

In Experiment 2, there was an effect of stallion ($p = 0.0062$), treatment ($p = 0.0001$), and treatment and stallion interaction ($p < 0.0001$) on sperm concentration. Pairwise comparisons indicated that the concentration immediately after collection was different ($p < 0.05$) than all treatment groups and there was no difference in sperm concentration between any treatment group. The mean sperm concentration for the combined treatments groups was 215.5 ± 3.4 x10^6 sperm/ml.

There was effect of collection ($p < 0.0001$), stallion ($p < 0.0001$), treatment ($p < 0.0001$), and collection and stallion interaction ($p < 0.0001$) on sperm viability. Sperm viability evaluated immediate post collection was higher ($p < 0.05$) than the treatment groups and there was no significant difference among treatments (Figure 2A).

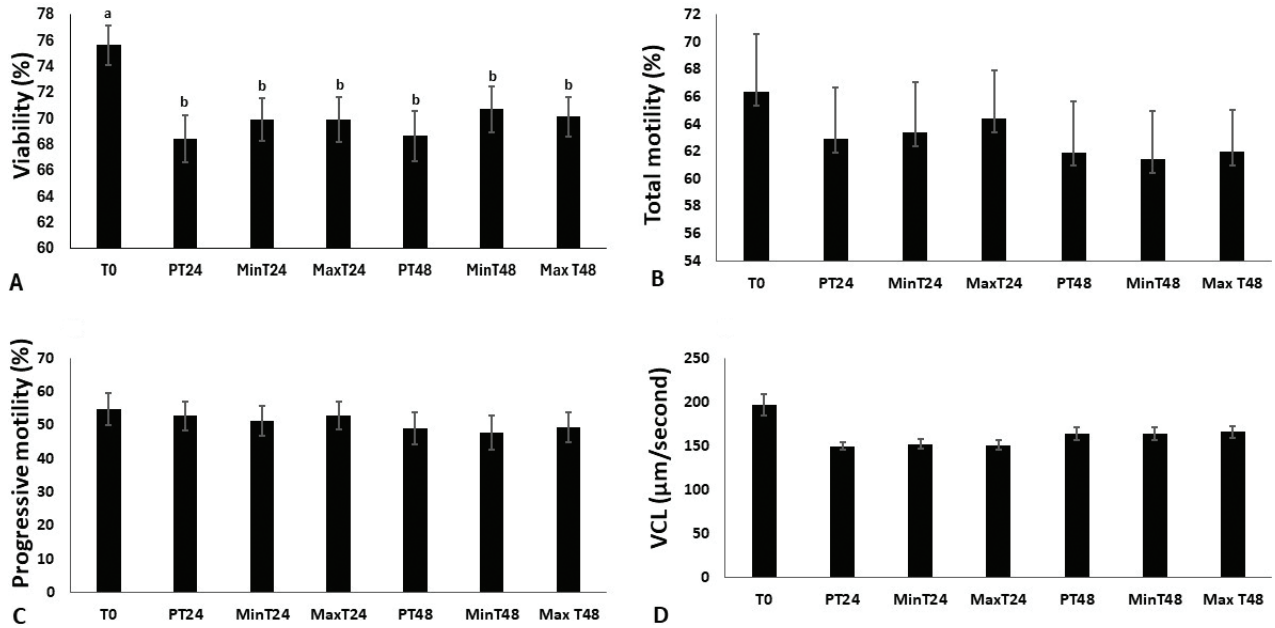

Figure 2. Percentage of sperm viability (A); total motility (B); progressive motility (C); curvilinear velocity (VCL µm/second) (D) at Time 0 (prior to centrifugation) and at 24 hours after mixing with a pipette (PT24); mixing at minimum vortex speed (MinT24) and maximum vortex speed (MaxT24), and 48 hours after mixing with a pipette (PT48); and mixing at minimum vortex speed (MinT48) and maximum vortex speed (MaxT48). Groups in A without common superscripts differed ($p < 0.05$).

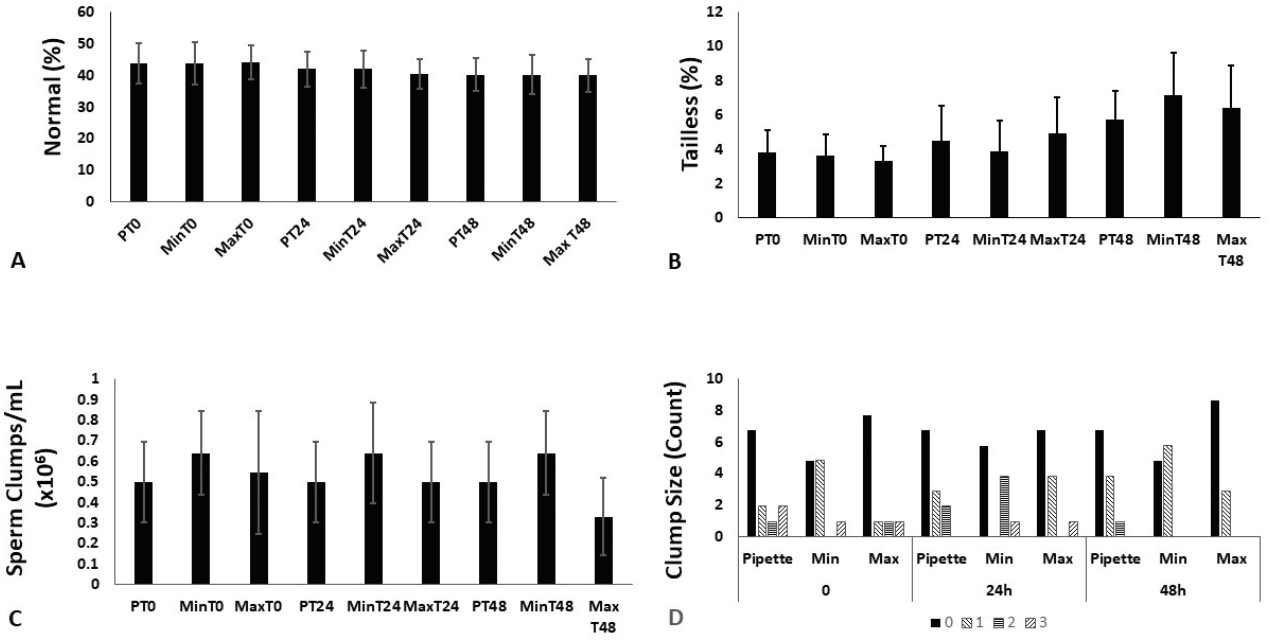

Figure 3. Percentage of morphologically normal sperm (A); number of tailless sperm (B); number of sperm clumps (x 10^6) per ml (C) and size of sperm clumps (classified as 1, 2, and 3) (D) at Time 0, 24, and 48 hours after mixing with a pipette (P); and mixing at minimum vortex speed (Min) and maximum vortex speed (Max).

There was effect ($p < 0.05$) of stallion and time ($p = 0.05$), and collection and stallion interaction ($p < 0.05$) on total and progressive motility. There was no treatment effect on total or progressive motility (Figures 2B and 2C).

There was effect of collection ($p = 0.0195$), stallion ($p = 0.0049$), and time ($p = 0.0008$), but no effect ($p = 0.95$) of treatment on VCL (Figure 2D). There was effect ($p = 0.005$) of collection, stallion ($p < 0.0001$), and time ($p = 0.0002$), and collection and stallion interaction ($p = 0.0189$) on STR.

There was effect ($p < 0.0001$) of collection, stallion ($p < 0.0001$), time ($p = 0.0075$), and collection and stallion interaction ($p < 0.0001$) on percent morphologically normal sperm (Figure 3A). There was effect ($p < 0.0001$) of stallion, time ($p = 0.002$), and stallion and time interaction ($p = 0.0015$) on the number of tailless heads (Figure 3B). There were no significant effects in the models for number of sperm clumps or size of sperm clumps (Figures 3C and 3D).

Discussion

To authors' knowledge, this is the first study to examine the effect of mixing technique on sperm quality, longevity, and clumping. There were no significant differences between mixing sperm via a pipette or vortex mixer after cushion centrifugation on sperm quality, longevity, or clumping.

There was no effect of treatment on motility, detached heads or size of sperm clumps but there was a difference in the number of sperm clumps among treatments (Experiment 1). Both treatments mixed with the pipette had the most sperm clumps suggesting that in this method mixing was not adequate. Increased number of pipette aspirations improved mixing; however, this still performed poorly compared to most vortex treatments. Therefore, we inferred that pipetting did not sufficiently agitate the solution compared to vortex mixing.

Vortex mixing in Experiment 1 had varying numbers of sperm clumps, depending on the vortex time, with vortexing for 5 seconds, regardless of speed, having the most clumps. At both the minimum and maximum vortex speeds, both 15 and 30 seconds had the fewest number of sperm clumps. Based on these data, both minimum and maximum vortex speeds for a 15 seconds vortex time were chosen for Experiment 2, since it performed better and was the shortest time that should minimize potential stresses on sperm.

There was no effect of treatment on sperm quality, longevity or sperm clumping (Experiment 2). These data suggested that mixing with either a vortex or pipette is a sufficient method after cushion centrifugation. The most interesting finding in Experiment 1 was a significant reduction in the mean number of sperm clumps among the pipette groups and some of the vortex groups; however, this was not observed in Experiment 2. This could be due to 2 factors; concentration of reconstituted semen and tube diameter. As concentration increases, the interparticular distance decreases, based on the equation: $C = \frac{1}{N\lambda^3}$, where N ≈ 6.02 x 10^{23} is Avogadro number and λ is the interparticle distance.[8] Thus, it seems reasonable to assume that after mixing a less concentrated solution there would be a lower likelihood of sperm interacting and forming aggregates (clumps) compared to a more concentrated solution. Secondly, a smaller diameter vortex (i.e. 15 ml conical tubes) has the material closer to the center of the vortex and consequently the material is stretched faster, reducing mixing time.[8] Thus, larger diameter tubes may require longer mixing times compared to smaller diameter tubes to have equal dispersion

Citation: Clinical Theriogenology 2024, 16, 10782, http://dx.doi.org/10.58292/CT.v16.10782

of sperm into a mixture. Thus, in hindsight, using 15 ml conical tubes in Experiment 1 to maximize the number of treatments per ejaculate, may not represent the ideal vortex times for a 50 ml conical tube to maximize mixing.

Fluid properties of protein solutions (which vary depending on the molecular weight and concentration of protein and type and concentration of liquid[3]) can alter the fluid stresses during mixing. Thus, various extenders could experience different stresses, depending on the composition, during mixing that could impact sperm quality. A chemically defined semen extender (INRA96)[9] was used in the present study. Thus, the findings herein reported are not necessarily applicable to other types of semen extenders, especially egg yolk based that have larger particles than skim milk based extenders.[10]

In summary, vortex mixing had the same impact on sperm (quality and longevity) as mixing with a pipette and may be a quick and applicable alternative for sperm pellet resuspension after centrifugation. Degree of clumping between 2 mixing methods varied with the concentration of sperm and size of the container used.

Conflict of interest

None to report.

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