Cushioned centrifugation improves recovery rate and viability of canine sperm

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Abstract

The objective was to compare recovery rate, motility and viability of canine sperm after cushioned and conventional (non-cushioned) centrifugation. Our hypothesis was that cushioned centrifugation improves sperm recovery rate relative to conventional, non-cushioned centrifugation without detrimental effects on sperm total motility or viability. Five adult dogs of varying breeds were collected weekly for 3 weeks. Each sample was processed, divided, and aliquots were centrifuged with and without cushion media. The aliquots were resuspended and analyzed for percent recovery, percent viability, and change in total motility. Cushion centrifugation had significant effects on recovery rate, and a numerical yet not statistically significant effect on total motility relative to conventional, non-cushion centrifugation of canine semen. Percent recovery was higher ($p = 0.041$) in cushioned centrifugation samples and a greater loss in viability ($p = 0.003$) was observed in non-cushioned centrifugation samples.

Keywords: Canine, cushion centrifugation, semen processing, sperm, semen quality

Introduction

Centrifugation is commonly done to remove excess canine seminal plasma. However, conventional, non-cushioned centrifugation techniques utilizing higher speeds (e 1620 x g) impose high mechanical forces, significantly decreasing sperm quality.[1] Lowering the centrifugation speed to d 720 x g helps prevent sperm damage, but decreases sperm recovery.[1] Since sperm concentration can vary with age and collection method, especially in older dogs, the practitioner's ability to adequately concentrate semen without damaging sperm becomes a clinical concern. Based on laboratory analysis in several studies, density gradient centrifugation of canine semen is effective in concentrating and selectively recovering higher quality sperm and is also capable of removing cellular contaminants.[2-4] However, effects of cushioned centrifugation alone on recovery rate and quality, as assessed by viability and motility of canine sperm, have apparently not been reported. Cushioned centrifugation of stallion semen effectively mitigated sperm damage and improved recovery rate.[5-7] Similar results were reported for boar semen.[8] Perhaps cushioned centrifugation would also be beneficial for dog semen.

The objective was to compare sperm recovery, motility, and viability of canine semen after cushioned and non-cushioned centrifugation. Our hypothesis was that cushioned centrifugation improves recovery rate without inducing significant detrimental effects on viability and motility of sperm relative to conventional, non-cushioned centrifugation.

Materials and Methods

Study design

Five adult dogs, aged 1.8 - 6.2 years (mean 4.8) were used. The breeds were Rhodesian Ridgeback, Chesapeake Bay Retriever, Saint Bernard, Great Dane, and Labrador Retriever cross. All dogs were privately owned and remained with their owners except during semen collection. Prior to acceptance into the study, each dog was subjected to a full physical examination and semen evaluation, and tested for brucellosis using a rapid benchtop assay (Anigen Rapid Canine Brucella Ab Test Kit, BioNote, Gyeonggi, Korea). The study protocol was approved by the Institutional Animal Care and Use Committee at Oklahoma State University. To reduce bias and increase consistency, only 2 individuals collected and processed semen samples, each one responsible for a unique set of tasks.

Sample collection and processing

A cleanout collection was performed 7 days prior to the first day of sample collection and processing in dogs (n = 5). Thereafter, 1 ejaculate was collected from each dog every 7 days for 3 weeks (total of 15 samples). To improve statistical power, each collection was regarded as an individual sample. No sexual activity was allowed between collections. Neat semen was analyzed for concentration and volume (determined by weight) to determine total sperm in the ejaculate. Sperm concentration and viability (live-dead ratio by propidium iodide exclusion) were evaluated using a NucleoCounter® SP-100™ (ChemoMetec A/S, Allerod, Denmark), according to manufacturer's instructions.

An aliquot of semen was then diluted to a concentration of 25 x 10^6 sperm/ml with canine-specific semen extender (CaniPro™ ApX2 Chill 5, Minitube of America, Verona, WI) to evaluate total motile sperm. All sperm motility samples were analyzed at a concentration of 25 x 10^6 sperm/ml following placement on a covered slide-warmer (37°C) for 10 minutes, using a computer assisted sperm motility analyzer (IVOS Version 12.0, Hamilton-Thorne Research, Hamilton, MA), as described.[9]

Extended semen containing identical amounts of sperm were placed into 2 conical vials (15 ml). In 1 of these 15 ml conical vials, 0.25 ml of cushion media (Cushion Fluid™, Minit b, Tiefenbach, Germany) was layered under the extended semen, as described.[5,6] The vial containing cushion media was then centrifuged at 1500 x g for 10 minutes, whereas the vial without cushion media was centrifuged at 750 x g for 10 minutes. The supernatant was removed via vacuum aspiration (Cook Medical, LLC, Bloomington, IN). For vials containing cushion media, a 7.62 cm spinal needle was inserted to the base of the vial in order to gently aspirate as much of the cushion media as possible without disrupting the sperm pellet layer. The volume of cushion media removed was minimal, totaling < 0.1 ml in each instance. The sperm pellet was suspended with extender containing 20% (v/v) of the subject's own fresh seminal plasma to 50 x 10^6 sperm/ml, as described.[6] Fresh seminal plasma was obtained by collecting and subjecting the third fraction of the ejaculate to centrifugation at 2000 x g for 10 minutes, followed by filtering the supernatant through tandem 5.0 and 1.2 µm nylon filters (Cameo 30 N Syringe Filter, Nylon, 30 mm; Sigma-Aldrich, St. Louis, MO) fitted to an all-plastic syringe to remove any remaining sperm. The sample was immediately analyzed for sperm recovery rate, viability, and total motility.

Statistical analyses

Analyses were performed for impact of treatment on sperm recovery rate, viability, and total motility. Recovery rate was calculated as ([sperm count/ml after treatment] / [sperm count/ml prior to treatment] x 100). Reduction in viability was calculated as [percentage of viable sperm prior to treatment – percentage viable sperm after treatment], and reduction in motility was calculated similarly [percentage of motile sperm prior to treatment – percentage of motile sperm after treatment].

A general linear model for repeated measures was done using commercial software (SPSS 21). Treatment (cushioned versus non-cushioned centrifugation) was considered a fixed effect. Main effects were considered only when no dog by treatment interaction was present.

Results

Percent Recovery

Mean percentage of sperm recovered post centrifugation was 98.7% (range 75.9 -128.7). Method of centrifugation affected ($p = 0.041$) percent recovery, with the cushioned centrifugation approach resulting in a larger percent recovery (103.5 versus 93.8%). There was no significant effect of dog on percent recovery and there was no treatment by dog interaction.

Percent Viability

Mean percent of viable sperm was 89.4% prior to centrifugation, 89.5% after cushioned centrifugation and 86.2% after non-cushioned centrifugation, with a difference among dogs ($p = 0.002$) and no treatment by dog interaction. There was a greater loss in viability ($p = 0.003$) in non-cushioned

versus cushioned approaches. Viability in cushioned samples was essentially unchanged when compared to diluted and non-centrifuged samples.

Percent Motility

Treatment had a highly variable impact on total motility and varied among samples from individual dogs. Changes in motility following centrifugation ranged from a decrease of 16% for a non-cushioned sample (90% initial motility down to 74%) to an increase in motility of 9% for 1 cushioned sample (69% initial motility to 78%). Mean changes were decreases of 3 and 6% for cushioned and non-cushioned centrifugation, respectively. No dog by treatment interaction was detected for change in total motility, and neither dog nor treatment affected motility following centrifugation ($p = 0.224$ and $p = 0.18$, respectively).

Discussion

In the present study, cushioned centrifugation improved sperm recovery rate when compared to conventional, non-cushioned centrifugation without negatively affecting viability of canine sperm. Results were consistent with those obtained in other domestic species.[5,7,8] However, there was no significant advantage in terms of percent loss of total motility. Given that the recovery rate of the cushioned samples was high, partial removal of the cushion media did not appear to negatively impact recovery rate in this study.

Higher recovery rate for the cushioned samples was attributed to an increased sperm sedimentation rate due to a higher centrifugal force, whereas non-cushioned samples were centrifuged at 720 x g to preserve viability.[1] In stallions, less conservative non-cushioned centrifugation protocols can yield good recovery rates without significant loss of sperm quality,[10] although such data have apparently not been reported for dogs. Perhaps canine sperm might remain viable with an acceptable recovery rate when centrifuged with moderate force even without the use of cushion medium (may require increased time). As viability can also be affected by increased duration of centrifugation, additional studies are needed to determine the optimal protocol if cushion centrifugation is to be avoided, for instance due to expense, unfamiliarity with the technique, or practitioner preference.

That average recovery rate for cushioned centrifugation samples was 103.5%, a value greater than what was present in the original sample, was unexpected. According to the manufacturer, the NucleoCounter® SP-100™ is accurate to within 4% on repeated analyses of the same sample for measurement of concentration, whereas accuracy for viability is repeatable to within 8%. Applying margins of error, recovery rate for the cushioned protocol would potentially be as low as 99.5% given the calculated average of 103.5%. However, since the initial (pre-centrifugation) measurements would also be subject to some degree of variation, original values may have been underestimated on average, thereby inflating subsequent readings. All analyses using the NucleoCounter® SP-100™ were performed by the same individual in order to mitigate interoperator differences.

The degree of change in viability varied among samples compared between dogs, but there was no interaction between a particular treatment and individual dogs. Perhaps sperm that responds poorly to conventional centrifugation still undergoes a noticeable loss of viability even with cushioned media. However, use of a cushion protocol reduced loss in viability, blunting a detrimental effect of the standard processing technique. The study population was comprised of dogs with normal initial semen quality based on published values with regard to sperm numbers, motility, viability, and morphology (data not shown).[11] Dogs with low sperm numbers or poor viability, such as aged or smaller breed dogs, may benefit the most from a cushioned centrifugation protocol to remove excess seminal plasma while minimizing damage to the sperm. A practical observation was that use of cushion fluid made dissolution of the sperm pellet subjectively easier and may have contributed to sustaining semen quality by reducing the vigor and time required to re-suspend sperm in solution.

The average effect on motility was a decrease in total motility relative to the initial, un-centrifuged samples. Although there was a numerical difference between the cushion and non-cushion protocols (decreases of 3 and 6% respectively), these were not statistically significant. This was consistent

with work done in the boar, although stallions have some variation. In that regard, there is not agreement in the literature as to whether or not centrifugation has a detrimental effect on stallion sperm motility.[8,9]

Based on the results of this study, no statement on fertility of the centrifuged canine sperm can be made. However, centrifugation is becoming more common for canine semen processing in practice and no significant detrimental effects would be expected in a dog with good initial semen quality when handled appropriately during processing. In stallions, incorporating small amounts of iodixanol cushion medium into the ejaculate during processing had no appreciable effect on semen quality or fertility.[5] The effects of incorporating cushion fluid into the ejaculate and its subsequent effects on fertility in the bitch are unknown. Further investigation with a fertility trial comparing non-centrifuged, cushioned centrifuged, and non-cushioned centrifuged semen is required to elucidate this effect.

Post-thaw motility and viability parameters of cryopreserved semen subjected to cushioned and non-cushioned centrifugation have yet to be compared. If sperm centrifuged without cushion were less likely to survive cryopreservation due to increased membrane damage, standard clinical procedures during semen cryopreservation would need to be altered.

In conclusion, cushioned centrifugation improved sperm recovery rate and maintained viability relative to conventional, non-cushioned centrifugation of canine semen. These findings supported the use of a cushion solution when performing centrifugation as a component of semen processing in dogs.

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Conflict of interest

The authors have no conflicts of interest.

References

1. Rijsselaere T, Van Soom A, Maes D, et al: Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 2002;57:1669-1681.
2. Dorado J, Alcaráz L, Duarte N, et al: Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 2011;76:381-385.
3. Phillips TC, Dhaliwal G, Verstegen JP: Separation of viable, motile sperm from red blood cells and dead spermatozoa: A comparison of four density gradient centrifugation media in the dog. Theriogenology 2008;70:578-579.
4. Hinshinuma M, Sekine J: Separation of canine epididymal spermatozoa by Percoll gradient centrifugation. Theriogenology 2004;61:365-372.
5. Waite JA, Love CC, Brinsko SP, et al: Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 2008;70:704-714.
6. Bliss SB, Voge JL, Hayden SS, et al: The impact of cushioned centrifugation protocols on semen quality of stallions. Theriogenology 2012;77:1232-1239.
7. Len JA, Beehan DP, Lyle SK, et al: Cushioned versus noncushioned centrifugation: Sperm recovery rate and integrity. Theriogenology 2013;80:648-653.
8. Matás C, Decuadro G, Martínez-Miró S, et al: Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 2007;67:1087-1091.
9. Schäfer-Somi S, Aurich C: Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. Anim Reprod Sci 2007;102:1-13.
10. Len JA, Jenkins JA, Eilts BE, et al: Immediate and delayed (after cooling) effects of centrifugation on equine sperm. Theriogenology 2010;73:225-231.
11. Johnston SD, Root Kustritz MVR, Olson PNS: Canine and Feline Theriogenology. New York: WB Saunders Company; 2001. p. 289.