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Preliminary Study of Effects of Centrifugation of Stallion Semen on Motility and Pro-AKAP4 Concentration over 72 Hours Post-collection

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Abstract: The study aimed to investigate the effects of centrifugation of stallion semen on progressive sperm motility over a 72-hour period; and determine if the protein marker, precursor of A-kinase anchor protein 4 (pro-AKAP4), may be maintained at a higher level over time when compared to non-centrifuged samples. Pro-AKAP4 has been studied for its function in stabilizing the structure of spermatozoa and its use as a marker for fertilizing capacity of spermatozoa. Semen collections were obtained from four fertile, commercially used Standardbred stallions collected three times each. Semen samples were diluted to a final concentration of 50 million total cells per milliliter. One extended aliquot of semen from each collection was used for the non-centrifuged treatment, and one extended aliquot of semen was centrifuged at 750 × *g* for 10 min. The supernatant from the centrifuged sample was then removed with a 10 mL plastic transfer pipette, and the spermatozoa cell pellet was suspended in fresh extender to a final concentration of 50 million total cells per milliliter. Both centrifuged and non-centrifuged samples from each collection were stored in Equine Express II stallion semen shipping containers for 72 h and fresh ice packs were added to each container every 24 h. Both progressive sperm motility and levels of pro-AKAP4 were determined at times 0, 12, 24, 48 and 72 h after collection. Differences were found between stallions, times and treatments with the main finding being that pro-AKAP4 concentration in centrifuged samples was maintained at a much higher level over the 72-hour period studied.

Key words: Centrifuged, extender, pro-AKAP4, spermatozoa, stallion.

1. Introduction

Semen consists of sperm cells and seminal plasma. Although seminal plasma likely contains components that are beneficial to spermatozoa under natural breeding conditions, it is not an ideal medium to cool and store spermatozoa [1]. Thus, centrifugation can be used to separate the sperm cells and the plasma. Centrifugation is the process of utilizing gravity to separate the seminal plasma of sperm from the semen based on varying levels of density to get concentrated spermatozoa cells isolated from the spermicidal effects of seminal plasma affecting the fertilizing portion of the sperm [2]. Centrifugation is thought to remove reactive oxygen species (ROS) and other elements that may cause sperm damage, allowing for a more balanced and beneficial medium for spermatozoal survival when suspended with semen extender and stored [3]. Semen extenders are utilized to maintain the motility of semen samples for more than 24 h [4]. The reduction of seminal plasma in extended semen allows sperm to have an increased tolerance to both cooling and freezing [5].

The most widely used method for breeding Standardbred horses is fresh chilled semen [6]. Centrifugation of extended stallion semen is becoming more common for the purposes of transferring samples or storing them for any length of time [1]. This method has been discovered to be a beneficial way of improving motility; however, there are potential deleterious effects associated with centrifugation [7]. Centrifugation leads to the loss of natural antioxidants in the seminal plasma and could result in a reduction in

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sperm quality and reduced sperm cell numbers [5].

The A-kinase anchor protein 4 (AKAP4) and its precursor (pro-AKAP4) have been investigated in various species and are thought to be a promising marker of stallion sperm quality [8]. AKAP4 expression is strictly in germ cells from the round spermatid stage until the mature spermatozoa [8]. AKAP4 is the most plentiful protein in the fibrous sheath, a cytoskeletal structure located in the principal piece of the sperm flagellum and constructed of 2 longitudinal columns connected by closely spaced circumferential ribs [9], and thus is instrumental in regulating sperm flagellum movement [8]. When AKAP4 is phosphorylated by tyrosine kinase, it generates a protein phosphorylation cascade that is involved in sperm motility and hypermotility [10]. It has been observed that a deletion in AKAP4 results in a defective fibrous sheath and flagellar dysplasia which results in infertility due to motility loss [8]. In a study on mice and AKAP4, the male mice lacking AKAP4 did not show a decline in sperm cell numbers; however, sperm failed to show progressive motility and were infertile [11]. In that same study, the males lacking AKAP4 exhibited formation of the sheath analog but the definitive sheath did not develop, the flagellum was shortened, and proteins typically associated with the fibrous sheath were absent or significantly reduced in the amount [11]. The other cytoskeleton components of the flagellum were present and correctly, fully developed [11]. Thus, AKAP4 is a major component of the fibrous sheath, which is responsible for supporting motility [12]. In a study investigating AKAP4 in human spermatozoa, pro-AKAP levels were found to be positively correlated with motility after density gradient centrifugation of the semen [13].

The aim of this study is to evaluate the changes in progressive motility of spermatozoa over a 72-hour storage period and the concentrations of the protein marker pro-AKAP4. The changes in progressive motility of spermatozoa over a 72-hour storage period were evaluated by recording observations of the percent motility of the samples at specific time points in that storage period, at the time of collection, 12 h after 24 h after, 48 h after, and then 72 h. The concentrations of the protein marker pro-AKAP4 were measured at those exact same time points utilizing an ELISA assay. Based on previous studies investigating the effects of centrifugation on progressive equine sperm motility over time, we hypothesized that centrifugation alone will significantly preserve the motility and fertilizing capacity of spermatozoa cells as evidenced by the protein marker Pro-AKAP4.

2. Materials and Methods

2.1 Stallion Semen Collections

Semen collections were obtained at the University of Illinois Horse Farm early in the morning during the off-breeding season in the months of October and November. Ejaculates were collected from four fertile Standardbred stallions three times each for a total of 12 ejaculates. Each stallion had been collected several times throughout the weeks leading up to the study, in order to minimize the number of senescent sperm cells stored in the epididymis. The Missouri model artificial vagina was utilized to collect the ejaculates as the stallions mounted a “phantom mare” (padded dummy). Once collected, semen samples were transferred into a sterile, plastic graduated container to measure the amount of ejaculate per collection per stallion.

2.2 Stallion Semen Processing

After recording the total semen volume initially collected, each sample was divided in half, one part undergoing centrifugation and the other part without centrifugation. The split sample to undergo centrifugation was initially diluted in a 1:1 ratio using Next Generation Dr. Kenney Semen Extender Plain without antibiotics during the centrifugation process and was centrifuged at 750× *g* for 10 min. The supernatant (seminal plasma and extender) was removed using a 10 mL plastic pipette and the sperm pellet was resuspended in INRA 96 extender. Both the centrifuged and

non-centrifuged samples were diluted with INRA 96 Stallion Semen Extender to a final concentration of 50 million total cells per milliliter. Our previous experience using the Dr. Kenny's extender for the centrifugation step only, found that we realized substantial economic savings when discarding the supernatant with a much less expensive extender, instead of discarding the supernatant with the much more expensive INRA 96, while still maintaining excellent motility over time when the sperm pellet was resuspended in INRA 96. Each sample was evaluated under an optical microscope at 400× magnification (40× objective lens and 10× ocular lens) by placing a single drop of semen on a pre-warmed slide and slide cover. Initial percent progressive motility was determined by two evaluators who were familiar with the microscopic assessment of stallion semen and documented progressive motility independently using two fields per sample for both centrifuged, and non-centrifuged. Each sample had at least 50% initial progressive motility, so the initial dilution rate yielded a desired initial progressive motile cell concentration of between 25-50 million progressively motile cells per milliliter.

Both treatments (centrifuged and non-centrifuged) were divided into 5 aliquots based on time points after collection: 0 h, 12 h, 24 h, 48 h, and 72 h. These aliquots were drawn up in 3 mL volume all-plastic syringes, taking care to remove all air bubbles and tightly cap each of the syringes, and the syringes were labeled for each time point to be analyzed. The labelled syringes were then placed into Equine Express II semen shipping containers with frozen ice packs on top of the syringes. The shipping containers were then placed into a refrigerator set at 4 ℃ and remained there throughout the 72-hour sampling period. The samples were evaluated for progressive motility at each of the time points. Each sample was evaluated after removing syringes from the shipping container. This was done by removing the cap from each syringe, drawing a small amount of air into each syringe in order to be able to gently invert each syringe to mix the cells, and placing a single drop of semen on a pre-warmed slide. After adding a pre-warmed slide cover, each sample was allowed to equilibrate for 5 min in an incubator set at 38 ℃. Two evaluators who were familiar with the microscopic assessment of stallion semen visually documented progressive motility independently using two fields on the microscope slide.

Immediately after placing the single drop of sperm on the pre-warmed slide, each syringe had all air bubbles removed once again, tightly capped, and put into a freezer at -18 ℃ to ensure that the sperm degradation process was halted at the proper time points following collection. Once all of the samples had the progressive motility documented at each time point, an ELISA assay to measure the pro-AKAP4 concentration of each sample at each time point was performed.

2.3 Pro-AKAP4 Determination

The concentration of the protein pro-AKAP4 in stallion sperm samples was quantified using the Horse 4MID ELISA kit manufactured by the 4BIODX/SPQI Company, France. The kit was composed of a 96-well plate and the required reagents to run the assay. A capture antibody coated the bottom of the plate that recognized the pro-AKAP4 protein. A detection antibody detected the capture antibody by covalently coupling to horseradish peroxidase. A substrate solution was added to each well that allowed the concentration of the protein present in each sperm sample to be proportionally displayed through varying color levels. Positive control was provided as well as standard solutions to run a linear standard curve and calculate the protein concentrations.

Three horse 4MID ELISA Kits were run to detect and quantify the protein marker pro-AKAP4 in each of the 120 samples: 4 stallions, 3 ejaculates per stallion, 2 different treatments, and 5 different time points. Duplicate sets of the 120 samples were run to detect possible outliers since only 88 samples could be analyzed on a single plate.

Linear regression was utilized to answer the question "Does centrifugation alone significantly preserve the motility and potential fertilizing capacity of stallions' spermatozoa over time?"

3. Results

A linear regression with percent progressive motility as response, horse name, time point, and treatment as predictors was performed. The fitted regression equation is as follows:

$$\% \text{ progressive motility} = 58.050 + 13.500 \times \text{Sirius} + 7.333 \times \text{Steve} + 17.500 \times \text{Viking} - 0.429 \times \text{Time} + 13.333 \times \text{Treatment}$$

Sirius = 1 if the horse name is Sirius, 0 otherwise, with Steve and Viking defined similarly (Sirius = Steve = Viking = 0 automatically implies the horse name is Sage).

Treatment = 1 if centrifuged, 0 if non-centrifuged.

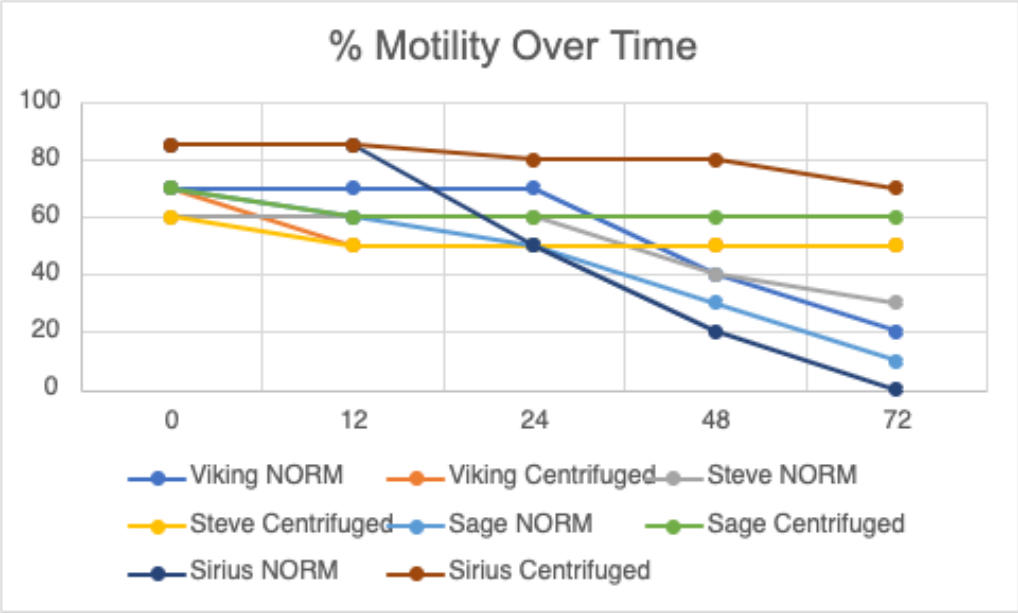

Fig. 1 The percent progressive motility values for each stallion's centrifuged and non-centrifuged (NORM) sample at each time points 0 h, 12 h, 24 h, 48 h, and 72 h.

Table 1 Analysis of variance summarizing the effect of treatment and other factors in percent progressive motility.

Variable	*F*-value	*p*-value	Conclusion
Horse	9.913	0	Highly significant
Time	83.601	0	Highly significant
Treatment (centrifuged/non-centrifuged)	30.224	0	Highly significant

The *F*-value is the value of the *F*-statistic that measures the effect of a predictor on the response. It can be observed that horse, time, and treatment have significant effects on the percent progressive motility.

Table 2 The *t*-tests performed to find which pairs of horses differ significantly in the percent progressive motility.

Horse 1	Horse 2	*p*-value	Adjusted *p*-value	Conclusion (based on adj. *p*-value)
Sage	Sirius	0.006	0.035	Significant
Sage	Steve	0.130	0.779	Not Significant (marginally)
Sirius	Steve	0.202	1.000	Not significant
Sage	Viking	0.000	0.002	Significant
Sirius	Viking	0.407	1.000	Not significant
Steve	Viking	0.037	0.219	Not significant

Only the pair Sage-Sirius is significantly different in terms of percent progressive motility.

Table 3 The *t*-tests performed to find which pairs of time points differ significantly in the percent progressive motility.

Time point 1 (h)	Time point 2 (h)	*p*-value	Adjusted *p*-value
0	12	0.333	1.000
0	24	0.033	0.325
0	48	0	0
0	72	0	0
12	24	0.235	1
12	48	0	0.007
12	72	0	0
24	48	0.023	0.234
24	72	0	0
48	72	0.049	0.492

There was no significant difference in terms of percent progressive motility in regards to the adjacent time points; however, as the time points get further away, the difference becomes highly significant between the two treatments. The centrifuged treatment has significantly higher percent progressive motility than the non-centrifuged treatment.

Since analysis of variance of differences in progressive motility due to horses, time and treatments found significant differences for each of these (Table 1), subsequent *t*-tests were done to compare differences in percent progressive motility due to horse (Table 2), time (Table 3), and treatment (Table 4). *t*-tests found that the centrifuged treatment had higher percent progressive motility than the non-centrifuged treatment. Since duplicate determinations of all samples were done in order to observe consistency of the pro-AKAP4 assay, a *t*-test was performed to find difference between duplicate assays of Pro-AKAP4 concentration (Table 5).

Because differences between duplicate assays of pro-AKAP4 were not found, either assay 1 or assay 2 could be used, and assay 1 was used for further comparisons.

A linear regression with the expression of the protein fertility marker pro-AKAP4 on assay 1 as response, horse name, time point, and treatment as predictors was performed. The fitted equation is as follows:

Expression of the protein marker of fertility pro-AKAP4 = -21.395 + 21.028 × Sirius + 38.7181 × Steve + 49.2232 × Viking + 0.160 × Time + 58.916 × Treatment

Sirius = 1 if the horse name is Sirius, 0 otherwise, with Steve and Viking defined similarly (Sirius = Steve = Viking = 0 automatically implies the horse name is Sage).

Treatment = 1 if centrifuged, 0 if non-centrifuged.

Analysis of variance summarizing the effect of treatment and other factors on concentration of Pro-AKAP4 found that both horse and treatment were significantly different (Table 6).

Since the stallions differed in levels of expression of pro-AKAP4, *t*-tests were performed in order to find out which pairs of stallions differed significantly (Table 7).

Since analysis of variance of differences between the two treatment effects found difference between centrifuged and non-centrifuged treatments a *t*-test determined that the centrifuged treatment had a significantly higher expression of pro-AKAP4 than the non-centrifuged treatment (Table 8).

Table 4 The *t*-test to compare difference between percent progressive motility of centrifuged versus non-centrifuged semen

Hypothesis	*p*-value	Conclusion
Centrifuged > non-centrifuged	0	Centrifuged values are significantly higher than non-centrifuged values

Not only does the centrifuged treatment have a different effect on percent progressive motility than the non-centrifuged treatment, but it is also significantly higher.

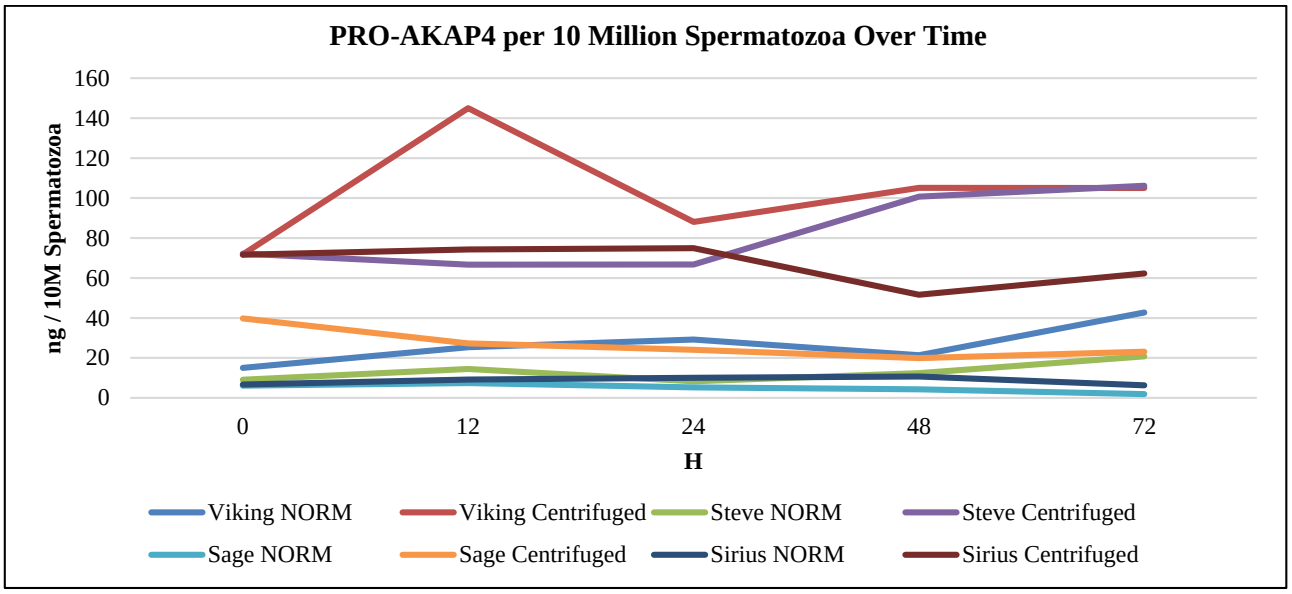

Fig. 2 The values for nanograms of pro-AKAP4 per 10×10^6 spermatozoa for each stallion's centrifuged and non-centrifuged (NORM) semen sample at each time point 0 h, 12 h, 24 h, 48 h, and 72 h.

Table 5 The *t*-test performed to find difference between duplicate assays of Pro-AKAP4 concentration.

Variable 1	Variable 2	Test	Test statistic value	*p*-value	Conclusion
Assay 1	Assay 2	Paired *t*-test	1.5127	0.133	No significant difference between the two assays.

Since there is no significant difference between the two assays, it is acceptable to work with either of them.

Table 6 An analysis of variance summarizing the effect of treatment and other factors on concentration of Pro-AKAP4.

Variable	*F*-value	*p*-value	Conclusion
Horse	8.756	0	Highly significant
Time	1.286	0.259	Not significant
Treatment (centrifuged/non-centrifuged)	65.329	0	Highly significant

The *F*-value is the value of the *F*-statistic that measures the effect of a predictor on the response. It can be observed that the different stallions and treatments have significant effects on the concentration of Pro-AKAP4, whereas the time did not.

Table 7 The *t*-tests performed to find difference in Pro-AKAP4 concentration between pairs of stallions.

Horse 1	Horse 2	*p*-value	Adjusted *p*-value	Conclusion (based on adj. *p*-value)
Sage	Sirius	0.105	0.629	Not significant
Sage	Steve	0.003	0.019	Significant
Sirius	Steve	0.172	1.000	Not significant
Sage	Viking	0.000	0.001	Significant
Sirius	Viking	0.030	0.182	Not significant
Steve	Viking	0.416	1.000	Not significant

Only the pairs Sage-Steve and Sage-Viking are significantly different in terms of expression of a protein marker of fertility.

Table 8 The t-test performed to find difference of Pro-AKAP4 concentration between centrifuged and non-centrifuged treatments.

Hypothesis	*p*-value	Conclusion
Centrifuged > non-centrifuged	0	Centrifuged values are significantly higher than non-centrifuged values.

Not only does the centrifuged treatment have a different effect on Pro-AKAP4 concentration over time the than the non-centrifuged treatment, but it is also significantly higher.

4. Discussion

Although there were some differences observed in the individual stallions' responses to the two different treatments (centrifuged and non-centrifuged), overall, the stallions' progressive motility improved at later time points following collection in response to the centrifugation, seminal plasma removal, and re-suspension of spermatozoa (Fig. 1). There was also improvement in the concentration of the protein marker of fertility pro-AKAP4 in the centrifuged samples when compared with the non-centrifuged samples for most stallions (Fig. 2). The differences between the individual stallions were primarily in the varying degrees of improvement.

Previous research has found that centrifugation of stallion semen can increase sperm cell longevity [6]. However, another study found that removal of seminal plasma worked mostly for stallions that were "bad coolers" [1], not for the good ones. Additionally, another study found that removal of seminal plasma did not affect motility, but significantly stabilized sperm membranes, as demonstrated by a higher response to the osmotic challenge semen, and might inhibit the acrosome reaction [14]. Research has found that the marker pro-AKAP4 is related to spermatozoa quality in regards to both sperm motility and fertility in human males [13]. This study is the first to look at how centrifugation of stallion semen affects the expression of the protein marker pro-AKAP4 following previous studies performed in the same lab that observed the effects of centrifugation and antioxidants on the motility of spermatozoa [6]. Prior studies of centrifugation of stallion semen have often found improved motility that supports this study's goal to further investigate possible reasons, or at least co-factors that may be related to progressive motility and fertility [1, 3, 6, 7]. This study further investigated stallion fertility by analyzing the effects of centrifugation on a protein marker pro-AKAP4 due to its putative usefulness as a marker for fertilizing capacity [8-11, 15, 16]. These findings suggest that centrifugation of stallion semen and seminal plasma removal may improve progressive motility of cooled, stored stallion semen over time, as well as maintain higher pro-AKAP4 concentrations. The small number of stallions used in the current study that were not previously characterized as either "poor or good coolers" makes strong conclusions difficult, and a cause-and-effect relationship between greater progressive motility over time and higher concentration of pro-AKAP4 in this study cannot be concluded. Still, the observation that centrifugation of stallion semen has a positive relationship with both longevity of progressive motility and the fertility marker protein, pro-AKAP4, offers a starting place for future research into how centrifugation of stallion semen may affect fertility measured by other variables such as pregnancy rates, covers per conception, live foal rates, etc. It is possible that the pro-AKAP4 is working as a marker of cell preservation or a cell protector itself, or as a reservoir of mature AKAP4 protein that might be activated as an alternative pathway to rescue sperm motility [13].

5. Conclusions

The results of this study found that centrifugation of stallion semen and removal of seminal plasma had a positive effect on the progressive motility of sperm over time as well as on the concentrations of pro-AKAP4 over time. The findings of this study suggest that centrifugation of stallion semen that will be used as fresh chilled semen over a period of up to 3 d may be done as a matter of routine processing in order to extend the useful life of the cool-stored semen of some stallions. Further studies with a large number of stallions are warranted to confirm this observation.

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