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Time Dependent Motility of Stallion Semen during Cool Storage Centrifuged in Two Different Extenders

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Abstract: The aim of this study was to evaluate the maintenance of spermatozoa motility over 72 h by two commercial semen extenders used to centrifuge semen before re-suspending the sperm pellet in INRA 96®. Centrifuged and re-suspended semen samples were evaluated for percent motility using four commercial Standard bred stallions collected three times each during the spring and summer breeding season. Next Generation Dr. Kenney's® without antibiotic or INRA 96® semen extenders were used to dilute semen samples in a 1:1 ratio prior to centrifugation at 750× *g* for 10 min. After decanting the supernatant, the resulting sperm pellets were then re-suspended and extended with INRA 96® to a final concentration of 50 million total cells per millimeter and stored in Equine Express II stallion semen shipping containers. Percent progressive motility was recorded at time points 0, 24, 48, and 72 h. Analysis of variance between stallions, time, and extender type found that there was no significant difference in percent motility at each of the time points between the two extenders ($p = 0.87$) used for centrifugation. Differences in percent motility over time and between stallions were found ($p < 0.001$). The findings suggest that the use of Next Generation Dr. Kenney's® semen extender during centrifugation and then re-suspending the sperm pellet in INRA 96® may be more cost effective than using INRA 96® for both centrifugation and re-suspension of the sperm pellet. This is due to the fact that the less expensive Next Generation Dr. Kenney's® supernatant is decanted and discarded after the centrifugation step, instead of the much more expensive INRA 96®, yet does not affect the quality of semen preservation and motility over time.

Key words: Semen, stallion, extender, centrifugation, motility.

1. Introduction

Artificial insemination is a common breeding method in most aspects of animal agriculture due to the convenience of using stored semen for local, regional, or international breeding of animals [1]. The equine industry in particular is a sector that heavily utilizes artificial insemination of either fresh cooled or frozen stallion semen. In addition to storage method, another factor that affects sperm motility is the use of semen extenders. Once the semen is collected from the stallion, the use of a semen extender not only increases the spermatozoa motility, but also the semen survival rate due to the minimization of bacterial contamination through the use of an extender containing antibiotics [2]. Common short-term semen extenders sold on the market today often include milk or egg yolk-based components [3]. While both extenders have been found to be effective in maintaining sperm motility over time, previous studies have indicated that milk-based extenders are more effective in maximizing progressive sperm motility over the course of 72 h [2]. A further step to maximize sperm longevity is using centrifugation, which separates the seminal plasma from the sperm cells. Centrifuging the semen has been found to preserve at least 75% of initial viable spermatozoa [4]. If not centrifuged, the sperm cell membrane may be destabilized, potentially reducing the spermatozoa lifespan [5]. Studies performed at the University of Illinois Horse Farm have consistently found that centrifugation of stallion semen has improved the longevity of stallion semen [6, 7]. The purpose of this study was to compare the effectiveness of two different semen extenders: INRA 96® and Next

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Generation Dr. Kenney's® extender in maintaining progressive motility over 72 h when used as the centrifugation media before being re-suspended in INRA 96®. We hypothesized that using the much less expensive Next Generation Dr. Kenney's® extender without antibiotics as the centrifugation media before re-suspending the sperm pellet in INRA 96® would be equally effective for maintaining progressive motility over time as using INRA 96® for both the centrifugation medium and re-suspension and storage of the cells over 72 h.

2. Materials and Methods

Semen samples were collected from four fertile commercial Standard bred stallions at the University of Illinois horse farm. The stallions were collected three times each over the course of a two-week period during breeding season. Each stallion was previously collected in order to "flush" the epididymi within two days prior to their collection date. Before collecting the samples, each stallion's penis was cleaned using warm water, paper towels and a cup for the final rinse. An ovariectomized mare was present for teasing the stallions before they mounted a phantom. All ejaculates were collected using a Missouri model artificial vagina and in-line cellulose filter to remove excess gel and any remaining contaminants. The filtered semen was then divided in half and placed into separate warmed plastic conical 50 mL centrifuge tubes. One tube was diluted in a 1:1 ratio with Next Generation Dr. Kenny's® semen extender without added antibiotics, and the other was diluted in a 1:1 ratio with INRA96® semen extender. Each extender was warmed to equine body temperature in an incubator prior to dilution. Sperm concentration for each collection was determined using a Densimeter®. Each 1:1 dilution of semen was placed in a conical tube and centrifuged for 10 min at 750× *g*. After centrifugation, the supernatant liquid was decanted into a waste container and fresh, warmed INRA96® was added to both conical containers. The cell pellets were diluted to a standardized concentration of 50 million cells per milliliter and the sperm pellets were carefully re-suspended into the liquid using a 10 mL plastic transfer pipette to gently agitate the cells by squeezing the bulb and directing liquid at the cell mass.

Aliquots from each treatment were drawn into 3 mL all plastic syringes and labeled according to treatment. Air was expelled from the syringe and capped to prevent contamination or exposure to air. One drop from the sample syringe was placed on a slide and put under a warmed microscope for motility evaluation at the initial time point (t = 0). Remaining syringe samples were then placed in an all-Styrofoam Equine Express II semen shipping container for cool storage. All samples were stored under ice packs which were changed daily to maintain storage temperature of 5 °C. Semen samples were evaluated for progressive motility at 0, 24, 48, and 72 h post-collection. Before evaluation, the plastic plungers were pulled back and the syringes were inverted to ensure thorough mixing. One drop of each sample was placed on a pre-warmed slide with a cover slip, and placed in the incubator to warm for 5 min before evaluation. To reduce the subjectivity of interpretation, at least two evaluators who were familiar with assessing progressive sperm motility under a microscope were present at each reading. Each evaluator was blinded to the estimate of the other, and values were averaged between evaluators. Used syringes were discarded in biohazard disposal containers after the slide was read and percent sperm motility was quantified. Statistical analyses, including linear regression and analysis of variance were done by the Illinois Statistics Office at the University of Illinois in Urbana-Champaign.

3. Results

Fig. 1 shows the mean percent progressive motility values and standard errors for all stallions at time points 0, 24, 48, and 72 h after semen was collected. Both treatments of initial centrifugation in either

INRA 96® or Next Generation Dr. Kenney's® showed similar reductions in progressive motility over time.

When the percent progressive motility was plotted on a histogram and a Shapiro-Wilk normality test was performed, the results were not normally distributed ($p < 0.05$) and slightly negatively skewed (Fig. 2). This is likely due to the differences in initial progressive motility of semen for each stallion used. Stallions were flushed within two days prior to collection and there was no limitation placed on initial progressive sperm motility. Some of the initial percent progressive motility values for stallions were as low as 50% or as high as 90%. Due to the non-normal distribution of data, a Box-Cox transformation was performed on the percent motility, where if $\lambda = 2$, the transformed variable became more normally distributed ($p = 0.05$) (Fig. 3).

A student's *t*-test was then performed to determine if there were differences in transformed percent semen motility over time between the two centrifuged semen extender types. The results indicate that there were no significant differences between the two extender types and percent motility over each time point (Table 1).

Multiple linear regression analysis was performed to test the effects of the individual stallion in comparison between the initial centrifuged semen extenders INRA96® and Next Generation Dr. Kenney's®. The estimated model equation used is as follows:

$$\text{Motility (\%)} = [2\times(2{,}318.25 - 339.58\times(\text{Stallion 1}) + 835.94\times(\text{Stallion 2}) + 873.44\times(\text{Stallion 3}) - 507.29\times(\text{Time 24 h}) - 881.77\times(\text{Time 48 h}) - 1{,}005.73\times(\text{Time 72 h}) - 16.15\times(\text{Kenny's})) + 1]^{1/2}$$

For each individual calculation, the stallion variable is 1 and other stallions are 0. Stallions 2 and 3 are similarly defined. If Stallions 1, 2, and 3 are 0, then the defined horse is Stallion 4 with a variable of 1. Time 24 h is 1 but 0 otherwise. Time 48 h and 72 h are similarly defined.

Analysis of variance was performed to compare means of the variables stallions, time points, and initial centrifuged semen extenders (Table 2). Individual stallions (*F*-value of 39.02) as well as each time point (*F*-value of 21.57) have highly significant effects on the percent motility whereas the type of extender used at centrifugation did not have a significant effect on the percent motility. The use of either Next Generation Dr. Kenney's® or INRA 96® semen extenders for centrifugation did not create a significant difference in percent motility of sperm over time for each of the stallions.

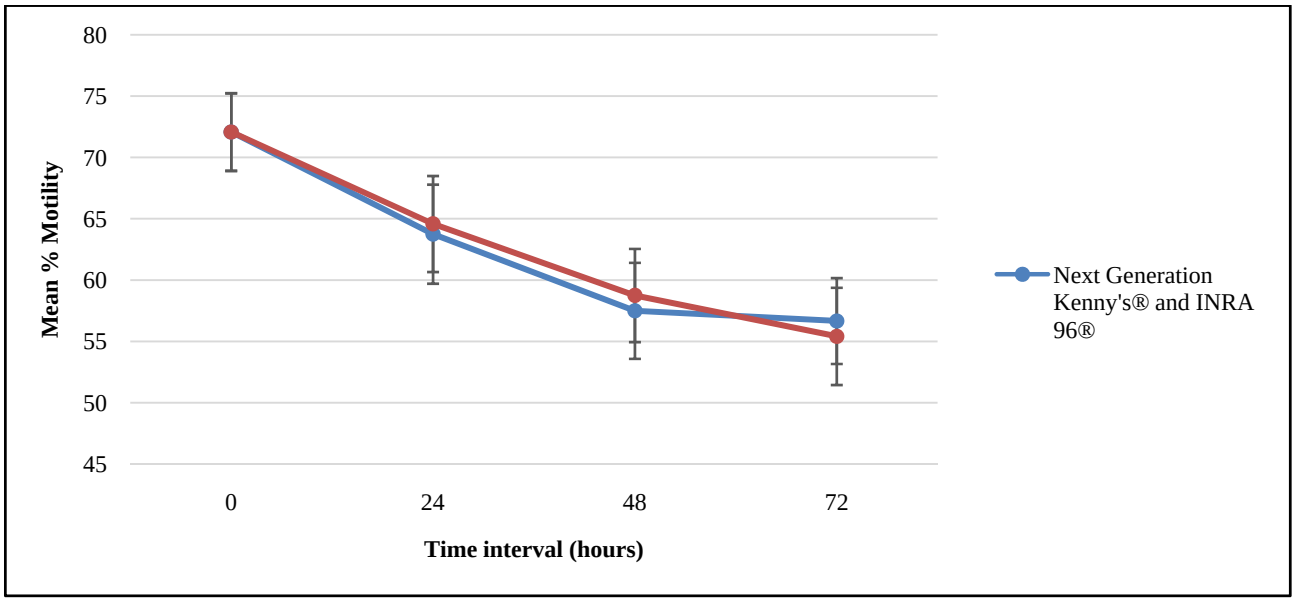

Fig. 1 Centrifuged semen extender means and standard errors of Stallions 1-4 over time.

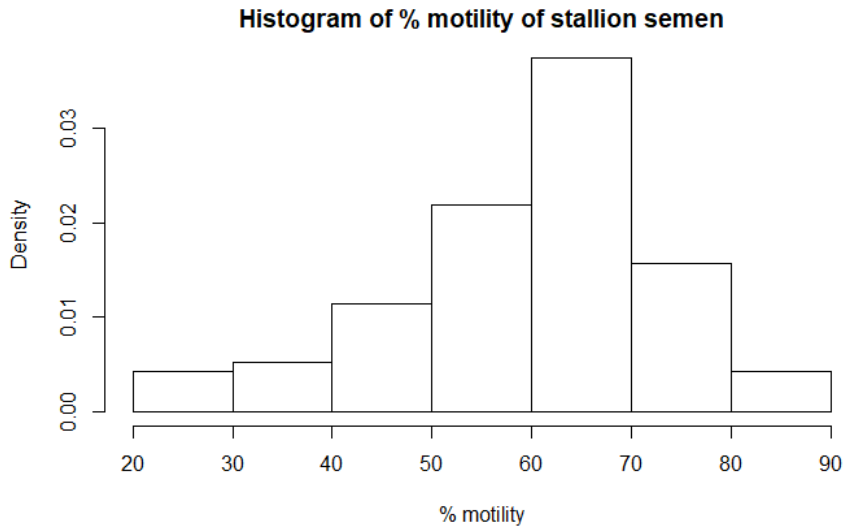

Fig. 2 Histogram of percent motility of stallion semen.

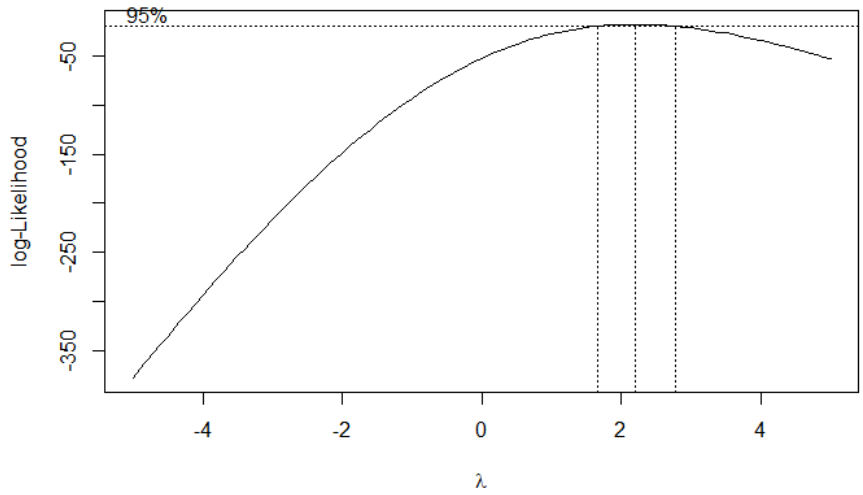

Fig. 3 Box-Cox transformation of percent motility.

Table 1 Student *t*-tests on transformed percent semen motility.

Time	*t*-value	Degree of freedom	*p*-value
All	0.098	94	0.922
0	0	22	1
24	0.145	22	0.886
48	0.241	22	0.811
72	-0.197	22	0.845

Table 2 Analysis of variance between means of stallions, time, and centrifuge extender type.

Variable	*F*-Value	*p*-value
Stallion	39.02	0
Time	21.57	0
Centrifuged extender	0.03	0.869

Table 3 ***t*-tests of pooled standard deviations between paired stallions.**

	Stallion 4	Stallion 1	Stallion 2
Stallion 1	0.35	—	—
Stallion 2	5.1×10^{-5}	1.3×10^{-8}	—
Stallion 3	2.2×10^{-5}	5.1×10^{-9}	1.00

Table 4 ***t*-tests of pooled standard deviations between time points (h).**

	0	24	48
24	0.09225	—	—
48	0.00026	0.42933	—
72	2.5×10^{5}	0.10319	1.00

Since stallions and time points had a significant effect on percent motility, pair wise comparisons were performed using *t*-tests with pooled standard deviations. Tables 3 and 4 show these differences between the pairs Stallions 2-4, Stallions 3-4, Stallions 2-1, and Stallions 3-1 as well as time points 0-48 h and 0-72 h. *p* values were adjusted using the Bonferroni method.

There was no significant difference in percent motility between the two centrifuged semen extenders Next Generation Dr. Kenney's® and INRA 96® at each time point (0-72 h). When the effects of each stallion and time points were tested using a multiple regression model, there was no significant effect of the centrifuged semen extender type on percent motility change. Individual stallions, however, had significant effects on the percent motility over time likely due to their varying ages and sperm motilities at the time of collection.

4. Discussion

Previous studies have found that diluted, centrifuged semen has reduced negative effects on semen quality due to seminal plasma contact with spermatozoa. After centrifugation, better sperm motility over time was found in stallions, boars and dogs [8-10]. Studies have also found that milk-based extenders, such as the Next Generation Dr. Kenney's® and INRA 96®, preserve sperm motility more effectively than egg yolk or sugar-based extenders when evaluating cool semen storage [2]. One study [11] concluded that centrifuging semen with either INRA 96® or another milk-based extender, BotuSemen, with cryopreservation extenders yielded overall higher sperm motility when examined after freezing for 24 h. In another study [12] that flushed the epididymis with an extender after thawing, motility results were similar between Next Generation Dr. Kenney's®, INRA 96®, and BotuSemen. Due to the cost-effectiveness and availability of Next Generation Dr. Kenney's® and industry-wide acceptance of INRA 96®, both were used in the current study for the centrifugation step only.

Although cool-stored semen is the typical choice for storage of equine semen for use in artificial insemination for just a few days, cryopreservation should also be considered when shipping semen over long distances as well as for storing semen from show or race horses that may not be available during breeding seasons. Freezing sperm can, however, be unfavorable due to the permanent damage to the cells caused by cold shock [13]. It should be noted that success of freezing semen depends on the cooling treatment prior to freezing as well as the individual stallion, since the most effective semen extender may not be the most effective for all stallions [14]. The addition of egg yolk products has been found to aid in preserving semen during the freezing process, but it is recommended to centrifuge the egg yolk to obtain the low-density lipoprotein (LDL) portion as the other components of the yolk can potentially interfere with cellular respiration and motility [15]. The use of

soybean lecithin in cryopreservation has become somewhat more popular because its lipid composition prevents ice crystal formation during the cold shock thawing process. There have also been growing concerns of microbial contamination from use of animal product based extenders [13]. The mean progressive motility for the centrifuged fresh chilled samples stored for as long as 72 h in this study exceeded 50%. Because of the relative vigor of the sperm cells that was maintained over a 3-day period, it might be useful to determine how well-preserved motility might be after freezing and thawing of semen from both treatments of the cooled semen stored at each of the time points. Only chilled fresh semen was evaluated in this study. However, evaluating both Next Generation Dr. Kenney's® and INRA 96® semen extenders to centrifuge and concentrate sperm cells before re-suspension and storage over multiple days prior to cryopreservation might reveal new ways to economically use stallion semen samples by reducing waste.

5. Conclusions

With the use of chilled fresh semen being a standard method for artificial insemination of equines, it is important to keep costs in mind when choosing a semen extender that will provide the best longevity of sperm motility in cool storage for 24 h or more. Previous studies have found that the use of milk-based extenders maximizes sperm motility of fresh chilled stallion semen over time when compared to egg based extenders. Studies have also concluded that centrifugation and re-suspension of the concentrated sperm pellet in fresh extender preserves greater motility when stored beyond 24 h, which is beneficial for shipped semen. The results of this study found that there was no significant difference in the percent motility changes over time when either INRA 96®, the more expensive semen extender, or Next Generation Dr. Kenney's®, the less expensive extender, were used initially during centrifugation. This suggests that it could be more cost effective to centrifuge semen samples in the Next Generation Dr. Kenney's® semen extender initially and then re-suspend the sperm in the INRA 96® for longer term chilled storage for breeding purposes over a period as long as 72 h.

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