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Motility of Stallion Spermatozoa after Centrifugation and Cooling in INRA96® or Walworth Extender

Gabrielle Nicolette Riccio[1], Robyn Ellienne Ellerbrock[2], Igor Frederico Canisso[2], Robert Victor Knox[1] and Kevin Halden Kline[1]

1. Department of Animal Sciences, University of Illinois Urbana-Champaign, 1207 West Gregory Drive, Urbana, IL 61801, USA

2. Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA

Abstract: A total of 18 ejaculates were collected, once per week, from six fertile stallions for three consecutive weeks in October and November, to compare motility over time between extenders using four semen processing treatments. Four total aliquots of semen were used. Two aliquots of each semen collection were extended in either INRA96® or an experimental proprietary milk-based extender Walworth (WW) extender, and each was designed for multi-day storage of fresh chilled semen. Each aliquot was divided again, and either centrifuged at 600 ×g for 10 min without cushion, or not centrifuged and extended to a final concentration of 25 × 10^6 spermatozoa/mL. The treatments evaluated were INRA96® without centrifugation (INRA-NC) or with centrifugation (INRA-C), and Walworth extender without centrifugation (WW-NC) or with centrifugation (WW-C). Total and progressive motility were measured using Sperm Vision® CASA at 0, 24, 48 and 72 h post-collection. Samples were stored at 4 °C. No differences were found between extenders in progressive (P = 0.13) or total motility (P = 0.14) over the four different time points without centrifugation. However, ejaculates processed in INRA-C group had the greater total and progressive motility (P < 0.05) over the four time points than ejaculates in the WW-C group. It was found that centrifugation and re-suspension of stallion semen in INRA96® improved the longevity of fresh chilled semen. However, when not using centrifugation, the Walworth extender proved to be as effective for maintaining spermatozoa motility across all time points as INRA96®, and may be an alternative for use in the equine breeding industry.

Key words: Stallion, semen, extender, centrifugation, fresh chilled.

1. Introduction

Fresh chilled stallion semen is most often preserved in milk-based extenders to provide nutrients, buffers and antibiotics needed to maintain fertilizing capacity long enough to inseminate Mares that may not be at the stallion's location. However, there are components in milk-based extenders that may also harm the semen, resulting in variable semen quality among extenders [1]. Semen quality parameters, including progressive and total motility have been found to be related to conception rates and embryo recovery rates [2]. The antimicrobials added to extenders, however, can reduce their effectiveness [3]. Extender performance may also be influenced by centrifugation, which is commonly used to concentrate spermatozoa in the semen of stallions with low cell concentration and/or seminal plasma that is toxic to the spermatozoa [4]. Centrifugation may improve membrane stability of spermatozoa [5]. Other methods of seminal plasma removal, such as semen filtration, have also been described [6]. Centrifugation of stallion semen may be done either with or without using cushioning material. In non-cushioned centrifugation, extended semen is centrifuged, the supernatant is then discarded and the sperm pellet at the bottom of the centrifuge tube is suspended in fresh extender. This method, however, can cause the dense pellet of non-discriminate cellular debris to be in close contact with sperm and cause damage [7]. It has been found that cushioned

Corresponding author: Kevin Halden Kline, Ph.D., research field: equine physiology.

centrifugation using several different combinations of sperm concentration and cushion fluid volume added to the centrifuge tubes before centrifugation resulted in no detrimental effect on initial or cool-stored sperm quality [8]. However, it has also been found that the optimal recovery rates preserving sperm integrity was obtained when semen was centrifuged at 900 ×g for 10 min without cushioning, as compared to semen centrifuged with cushion at either 900 ×g or 1,800 ×g for 10 min [9]. Additionally, there may be differences between the effectiveness of dry milk-based extenders and liquid; however, because both of the extenders used in the current study were liquid, this is not a confounding factor in this study [10]. It was hypothesized that fresh stallion semen that was either non-centrifuged or centrifuged without cushion material and extended in either INRA96® or a proprietary experimental milk-based extender, would not differ in progressive or total motility when stored at 4 °C over 72 h. The purpose of the study was to assess two different extenders to determine if using non-cushioned centrifugation improved sperm fertility of extenders with different compositions. An additional objective of this study was to assess if centrifugation prolonged spermatozoa motility after the 72 h in storage. If centrifugation provides better spermatozoa motility past the 72 h time point compared to non-centrifugation, these data would be useful in mare insemination protocols over prolonged periods.

2. Materials and Methods

Eighteen ejaculates (n = 18) were collected from six reproductively healthy light breed stallions ranging in 8-17 years of age. Each stallion was collected three times from October to November over a period of three weeks. Ejaculates from the six stallions were handled separately and not pooled. Initial sperm concentration was determined using an Equine Densimeter®, and then the ejaculates were split into four aliquots. The first aliquot was extended 1:1 in INRA96®, and the second was extended 1:1 in the Walworth extender. Both were centrifuged at 600 ×g for 10 min. After centrifugation, all but 5 mL of the supernatant was removed, and the sperm pellet was re-suspended with 10 mL of the INRA96® or Walworth extender. Sperm concentration was then assessed with a hemocytometer, and the aliquots were further extended to reach a final concentration of 25 × 10^6 million spermatozoa/mL. The third and fourth aliquots were also extended to 25 × 10^6 million spermatozoa/mL in INRA96® or the Walworth extender, but were not centrifuged. Evaluated treatments were INRA96® without centrifugation (INRA-NC) or with centrifugation (INRA-C), and Walworth extender without centrifugation (WW-NC) or with centrifugation (WW-C). Each sample was then split into three aliquots and packaged anaerobically in Whirl-Paks for evaluation at 24, 48 or 72 h. All samples were stored in an Equitainer® at 4 °C before evaluation. After 24 h, samples were warmed in an incubator to 37 °C for 10 min before analysis. This was repeated for the second aliquot at 48 h and the third at 72 h. The specific gravity for each extender was determined at 29 °C using a glass hydrometer to detect any obvious differences in overall solute concentrations between extenders.

Total and progressive motility were measured using Sperm Vision® computer assisted sperm analysis at 0, 24, 48 and 72 h post-collection. Each collection was warmed in an incubator at 37 °C for 10 min before analysis. Aliquots (10 µL) were placed on a pre-warmed slide with a coverslip. Total and progressive motility were recorded for 10 fields counting 500 sperm per field.

Data were modeled as repeated measures over time. The statistical analysis was conducted using the mixed procedure of SAS® (Version 9.4) and significant differences were identified using t-tests of least squares means differences. Statistical significance was set at $P < 0.05$. The adjustment used was the Tukey-Kramer test. Data were expressed as means ±

standard error of mean (SEM).

3. Results

Total and progressive motility values for all time points for all treatment groups are shown in Tables 1 and 2. Table 1 shows that total motility in both extenders declined by about 30% from 0 h through 72 h of storage at 4 °C when not centrifuged. However, when the INRA96® samples were centrifuged, the total motility after 72 h of storage had declined by only about 18%, while the centrifuged Walworth samples had declined in total motility by about 27%. Table 2 shows that progressive motility in both extenders declined by about 52% from 0 h through 72 h of storage at 4 °C when not centrifuged. However, when the INRA96® samples were centrifuged, the progressive motility after 72 h of storage had declined by only about 30%, while the centrifuged Walworth samples had declined in progressive motility by about 41%. The total and progressive motility before centrifugation were similar for both INRA96® and the Walworth extender at all time points ($P > 0.05$). After centrifugation, there was a difference in the INRA-C and WW-C ($P = 0.008$) at the 24 h time point and continued through the 72 h time point. The total and progressive motility means were higher for INRA-C than that for WW-C at 24, 48 and 72 h. Specific gravity determinations found that INRA96® had a specific gravity of 1.05 and the Walworth extender's specific gravity was 1.04, thus the concentration of total solutes was very similar between the extenders.

4. Discussion

INRA96® has previously been proven to be an effective multi-day fresh cooled stallion semen extender for up to 72 h [4]. The current study not only evaluated INRA96® as a medium-term extender for up to 72 h, but also its effectiveness in maintaining total and progressive motility post centrifugation, and compared its performance with the proprietary Walworth extender. Although the specific ingredients for the Walworth extender were not made available due to the proprietary nature of the product, the manufacturers indicated that the product included

Table 1 Mean percent of total sperm motility (TM) in freshly chilled semen from three ejaculates collected from six reproductively healthy stallions (n = 18) under different treatments at four time points following semen collection.

Time (h)	TM ± SEM (%)			
	INRA-NC	INRA-C	WW-NC	WW-C
0	84.73 ± 2.50[a]	89.64 ± 1.90[a]	85.27 ± 2.00[a]	86.49 ± 2.90[a]
24	74.70 ± 3.50[a]	82.82 ± 3.30[b]	70.42 ± 4.70[a]	75.09 ± 3.20[a]
48	66.78 ± 4.80[a]	81.42 ± 2.70[b]	66.29 ± 4.60[a]	67.58 ± 2.00[a]
72	60.71 ± 4.40[a]	74.39 ± 4.40[b]	59.75 ± 4.50[a]	63.51 ± 5.90[a]
Overall means	71.73 ± 3.74[a]	81.97 ± 3.70[b]	70.43 ± 3.74[a]	73.17 ± 3.74[a]

Each collection was processed with INRA96® or Walworth extender and with (C) or without centrifugation (NC).
SEM: standard error of mean; values within a row with different superscripts are statistically significant ($P < 0.05$).

Table 2 Mean percent of progressive sperm motility (PM) in freshly chilled semen from three ejaculates collected from six reproductively healthy stallions (n = 18) under different treatments at four time points following semen collection.

Time (h)	PM ± SEM (%)			
	INRA-NC	INRA-C	WW-NC	WW-C
0	75.84 ± 4.00[a]	82.65 ± 2.70[b]	77.76 ± 2.50[a]	78.67 ± 3.80[a]
24	58.64 ± 6.00[a]	72.70 ± 4.8[b]	54.85 ± 5.50[a]	61.24 ± 4.80[a]
48	48.14 ± 5.80[a]	69.14 ± 4.30[b]	47.90 ± 6.80[a]	45.46 ± 5.00[a]
72	36.40 ± 5.40[a]	58.00 ± 5.50[b]	37.83 ± 5.80[a]	46.10 ± 6.10[a]
Overall means	54.76 ± 5.23[a]	70.62 ± 5.23[b]	54.59 ± 5.23[a]	57.87 ± 5.23[a]

Each collection was processed with INRA96® or Walworth extender and with (C) or without centrifugation (NC).
SEM: standard error of mean; values within a row with different superscripts are statistically different ($P < 0.05$).

ingredients that bind and coat the sperm cells. The formulation was intended to slow spermatozoa mitochondrial metabolism and reduce the speed of motility while extending the duration of motility, in addition to providing acrosome protection. This experiment was not designed to verify these claims, but rather to compare the experimental product to the well-known INRA96® with regard to motility percent over time. However, visual inspection of spermatozoa under a microscope extended in the Walworth extender or INRA96® confirmed that the speed of motility in freshly extended semen was apparently slower in the Walworth extender as compared to the INRA96®.

The use of centrifugation improved the progressive and total motility of spermatozoa extended in INRA96® over time, but not for the Walworth extender. Maintenance of total and progressive motility over time was not different between the INRA96® and Walworth extenders without centrifugation. It is possible that the effects of centrifugation on total and progressive motility of spermatozoa stored in the two extenders may have been different, if cushion fluid or different centrifugation speeds had been used, but those treatments were not evaluated in this study. Greater motility may have been achieved by using a previously evaluated centrifugal force and time combination of 500 ×g for 10 min [3]. Although the centrifugal force used in this study was not much greater, the maximum recovery rate may have been achieved at a lower speed. The differences between extenders found in this study related to centrifugation were not likely due to differences in specific gravity, because the similar values were found for both extenders (1.04 for the Walworth extender versus 1.05 for the INRA extender). It was found that stallion semen centrifuged and re-suspended in INRA96® had the greater longevity than that centrifuged and re-suspended in the Walworth extender. However, when not using centrifugation, the Walworth extender proved to be as effective for maintaining spermatozoa motility across all time points as INRA96®. This finding may be more relevant if the Walworth extender proves to be less expensive than INRA96®; however, the new product is not yet in the commercial marketplace, so this comparison can not currently be made. The findings of this study suggest that the Walworth extender may be an acceptable alternative to INRA96® for equine breeding operations that may not have or do not choose the use of a centrifuge, assuming that the market price of the new product is competitive with INRA96®.

5. Conclusions

The study results found that stallion semen centrifuged and re-suspended in INRA96® had the greater longevity than that centrifuged and re-suspended in the Walworth extender. When not using centrifugation, Walworth extender proved to be as effective for maintaining spermatozoa motility across all time points as INRA96®. Walworth extender is an acceptable alternative to INRA96® for equine breeding operations that do not use centrifugation for stallion semen processing.

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