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SHORT COMMUNICATION

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Stallion sperm quality after combined ejaculate fractionation and colloidal centrifugation

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Abstract

This study investigated the possible additive benefit of ejaculate fractionation and colloidal centrifugation on stallion sperm quality. Using an open-end artificial vagina, the sperm-rich fraction (FRAC-1) was separated from the rest of the ejaculate (FRAC-2) and a third sperm sample representing the combined ejaculate was reconstituted post-ejaculation (RAW). Each semen sample was processed for colloidal centrifugation. The percentage of abnormal spermatozoa was 17.8 ± 7.0% in RAW and 14.6 ± 9.5% in FRAC-1 but decreased to 11.4 ± 4.7% and 9.6 ± 6.9% respectively, after colloidal centrifugation. A sperm DNA fragmentation index of 10.9 ± 5.1% was observed in RAW and 7.5 ± 2.4% in FRAC-1 semen collected with the AV but this decreased to 7.8 ± 2.8% and 5.2 ± 2.3% after colloidal centrifugation. The rate of increase in sperm DNA fragmentation during the first 6 h of incubation at 37 ºC was 1.8 ± 0.9% per hour in RAW semen and 2.0 ± 2.0% per hour in FRAC-1 but this significantly decreased to 1.3 ± 1.4% and 0.9 ± 0.8% respectively after colloidal centrifugation. While stallion seminal characteristics can be improved using colloidal centrifugation, further enhancement is possible if the ejaculate is initially fractionated.

Additional key words: *Equus caballus*; ejaculate fractionation; colloidal centrifugation; sperm DNA fragmentation; DNA longevity; SCD.

Abbreviations used: FRAC-1 (first jet, sperm-rich fraction); FRAC-2 (rest of the ejaculate); RAW (combined ejaculate reconstituted post-ejaculation); ROS (reactive oxygen species); rSDF (rate of increase in sperm DNA fragmentation); SCD (sperm chromatin dispersion), SDF (sperm DNA fragmentation).

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Single layer colloidal centrifugation of the stallion ejaculate has been shown to result in the selection of the most robust spermatozoa possessing intact and mature chromatin (Johannisson *et al*., 2009; Morrell *et al*., 2009a, 2014b; Gutiérrez-Cepeda *et al*., 2011). In addition, potential DNA damage associated with reactive oxygen species (ROS) can be prevented by removing damaged or dead spermatozoa and leukocytes by means of colloidal centrifugation, thus further contributing to maintenance of sperm chromatin integrity (Johannisson *et al*., 2009; Morrell *et al*., 2009a,c). Colloidal centrifugation can also help to eliminate pathogens from the semen sample (Loomis, 2006; Ortega-Ferrusola *et al*., 2009; Morrell & Wallgren, 2011) but the efficiency of the removal of bacteria appears to be dependent on both the species and the respective bacterial load (Morrell *et al*., 2014a). Moreover, single layer colloidal selected spermatozoa show normal function when injected into equine oocytes (Colleoni *et al*., 2011) and after artificial insemination; even after storage for 96-hours these spermatozoa can result in high pregnancy rates (Lindahl *et al*., 2012). Gutiérrez-Cepeda *et al*. (2012) have also reported that single layer colloidal centrifugation performed prior to cryopreservation reduces DNA fragmentation during the first four hours after thawing following incubating at 37 °C. The efficiency of single layer colloidal centrifugation in selecting robust stallion spermatozoa from frozen-thawed semen has likewise been reported (Macías-García *et al*., 2009).

Despite the efficiency and utility of colloidal centrifugation, there are other strategies that can be used that do not require a direct intervention or treatment of the semen sample but where a physiological reduction in the level of sperm DNA damage can still be obtained. For example, in those species which exhibit a distinct fractionation of the ejaculate, such as the stallion, a simple ejaculate partitioning technique can be conducted to separate the first and the second fractions leading to improvements in semen quality when compared to raw semen (Kumar *et al*., 2011; Valsa *et al*., 2012). Although some ejaculate parameters, such as seminal volume may decrease, other more relevant characteristics, such as sperm concentration or motility may be improved by implementing this sperm recovery strategy (Sieme *et al*., 2004; Kareskoski *et al*., 2011b). If fractionation of the stallion ejaculate leads to better semen quality, then we propose that this technique has the potential to be further enhanced by single colloidal centrifugation (Johannisson *et al*., 2009; Morrell & Rodríguez-Martínez, 2009; Morrell *et al*., 2010b). The present study aimed to investigate the potential additive benefits on sperm quality that might result from combining single-layer colloidal centrifugation with fractionated semen.

Ejaculates were obtained from twelve different stallions of Spanish pure breeds at the Centro Militar de Cría Caballar in Ávila (40.66ºN 4.70ºW) during the breeding season. Stallions ranging in age from 7 to 15 years, that were unrelated and clinically healthy, were chosen for this study. The stallions mounted a phantom and ejaculated into an open-end artificial vagina (Missouri–Nasco, Fort Atkinson, WI, USA) which was used for ejaculate fractionation; this consisted of a modified Polish or Krakow model AV, whereby the distal collecting cone was removed. The sperm rich fraction (first jet: FRAC-1) was separated from the rest of the ejaculate (FRAC-2). For experimental purposes, a third semen sample (FRAC-1 and FRAC-2) was promptly reconstituted post-ejaculation in order to imitate the sperm characteristics of the whole complete ejaculate (RAW). This reconstituted RAW sample was then promptly filtered to remove the gel fraction. Sperm concentration was measured in both semen fractions and the reconstituted RAW sample, which were immediately extended 1:1 in INRA 96® (IMV Technologies, lÁigle, France) and kept at 37 ºC for assessment of motility (total and progressive), sperm morphology and sperm DNA integrity.

One aliquot of FRAC-1 and of the reconstituted RAW sample were processed by centrifugation through a single layer colloidal, which was prepared by pipetting a 5 mL volume of the higher density layer (80% bottom layer Equipure™; Nidacon International AD Mölndal, Sweden) into a 15 mL tube and carefully adding another 5 mL volume of the extended semen on top. After loading, the tubes were centrifuged at 300 g for 20 min (Gutiérrez-Cepeda *et al*., 2011) and the supernatant and the majority of the colloidal material removed by aspiration post centrifugation. The resulting sperm pellet was then diluted in INRA 96® and evaluated for sperm characteristics. Sperm concentration and motility were assessed using a computer-assisted sperm motion analyzer microscope (Sperm Class Analyzer®, Microptic SL, Barcelona, Spain). The percentage of sperm with abnormal morphology was determined by evaluation of 100 cells after fixing an aliquot of each sperm sample in buffered formalin (Varner, 2008). Sperm DNA fragmentation (SDF) was assessed using the Halomax kit® (Halotech SL, Madrid, Spain). This methodology is based on the sperm chromatin dispersion test (SCD), the details of which have previously been described in a range of mammalian species, and which had already been validated for equine semen by our group (López-Fernández *et al*., 2007). Sperm DNA longevity in the different fractions was calculated after a dynamic SDF assessment protocol which was designed to emulate conditions of sperm physiology once inside female reproductive tract. For this purpose, an aliquot of each sperm sample (FRAC-1, FRAC-2 and RAW) was extended in INRA 96® (IMV Technologies, lÁigle, France) to provide a semen concentration of around 25 million/mL and incubated for 24 h at 37 ºC to perform SDF assessment at time 0 (immediately after sperm recovery and dilution; SDF-T0), after 6 h (SDF-T6) and after 24 h (SDF-T24) of incubation. For each experiment, 25 µL of diluted spermatozoa (10 million/mL) was added to a vial containing low melting point agarose and gently mixed. A small aliquot of the agarose-sperm mixture (10 µL) was then spread upon pre-treated slides (provided in the Halomax® kit), covered with a glass coverslip and placed in a refrigerator on a cold metallic plate for 5 min. Following solidification, the coverslip was carefully removed and the 'spermgel' slide preparation placed horizontally in 10 mL of the lysing solution provided in the Halomax® kit for 5 min. The 'sperm-gel' preparation was subsequently washed in dH_2O for 5 min and then dehydrated in a series of ethanol baths (70%, 90% and 100%). For direct visualization of DNA damage, fluorescence microscopy was conducted using the dual emission DNA-red/protein-green fluorochrome combination provided in the Halomax® kit. This combination of fluorochromes allowed selective staining of DNA in the head of the sperm (red) and the flagellum and the sperm core (green). Three hundred spermatozoa from each incubation time at 37 °C were counted to estimate SDF at each time in-

terval. The rate of increase in SDF was calculated from T0 to T6 (rSDF-T6) by subtracting SDF values of the two consecutive intervals (SDF-T6 minus SDF-T0) and dividing the result by the 6 h elapsed time. Statistical analysis was performed using the Statistical Package for the Social Sciences 17 (SPSS Inc., Chicago, IL, USA). A non-parametric statistical Wilcoxon signed-rank test was used for comparisons between two related samples. Significance was set at $p \leq 0.05$.

Table 1 summarizes the mean values and standard deviations for the range of sperm characteristics analysed from the different fractions and RAW semen. Table 2 summarizes the statistical analysis of multiple Wilcoxon signed-rank tests that we have used to compare two related samples. In the first analysis, we compared uncentrifuged RAW semen to uncentrifuged FRAC-1 and colloidal centrifuged RAW semen, respectively. A significant improvement was obtained after colloidal centrifugation of RAW semen in terms of progressive motility, abnormal morphology sperm, and sperm DNA stability (as shown by both SDF-T0 and rSDF-T6). After ejaculate fractionation, we obtained a significantly higher sperm concentration in FRAC-1, which also contained sperm samples with significantly higher total motility and lower SDF-T0 (Tables 1 and 2). Secondly, we compared uncentrifuged FRAC-1 and colloidal centrifuged RAW semen, to explore whether an improvement in seminal characteristics could be achieved using ejaculate fractionation over colloidal centrifugation (Table 2). Only concentra-

Table 1. Mean (± standard deviations) of seminal characteristics for each fraction and RAW semen before and after colloidal centrifugation (sample size N=12). CONC: concentration, TM: total motility, PM: progressive motility, ABM: percentage of abnormal morphology sperm, SDF-T0: percentage of fragmented sperm at T0, rSDF-T6: rate of sperm DNA fragmentation from T0 to T6. Concentration is expressed in millions/mL. All other variables are expressed as percentage.

	CONC	TM	PM	ABM	SDF-T0	rSDF-T6
Uncentrifuged						
FRAC-1	349.7 ± 114.9	84.9 ± 8.1	54.1 ± 8.2	14.6 ± 9.5 (a)	7.5 ± 2.4 (f)	2.0 ± 2.0
FRAC-2	135.7 ± 49.2	71.4 ± 14.1	40.6 ± 12.9	20.8 ± 6.1 (b)	21.4 ± 19.5 (g)	3.4 ± 2.9
RAW	257.3 ± 74.8	78.7 ± 10.7	49.8 ± 13.2	17.8 ± 7.0 (c)	10.9 ± 5.1 (h)	1.8 ± 0.9
Colloidal centrifuged						
FRAC-1	198.9 ± 80	87.0 ± 6.6	59.0 ± 9.2	9.6 ± 6.9 (d)	5.2 ± 2.3 (i)	0.9 ± 0.8
RAW	141.3 ± 45.8	82.0 ± 11.8	55.7 ± 10.8	11.4 ± 4.7 (e)	7.8 ± 2.8 (j)	1.3 ± 1.4

(a) 175 out of 1200 cells, (b) 250 out of 1200 cells, (c) 214 out of 1200 cells, (d) 115 out of 1200 cells, (e) 137 out of 1200 cells, (f) 270 out of 3600 cells, (g) 771 out of 3600 cells, (h) 393 out of 3600 cells, (i) 186 out of 3600 cells, (j) 279 out of 3600 cells.

Table 2. Results of the Wilcoxon signed-rank test for the different comparisons and seminal characteristics (sample size is N=12 for all comparisons). Wilcoxon´s Z and *p*-values (in parentheses) are shown for each comparison. CONC: concentration, TM: total motility, PM: progressive motility, ABM: percentage of abnormal morphology sperm, SDF-T0: percentage of fragmented sperm at T0, rSDF-T6: rate of sperm DNA fragmentation from T0 to T6.

Comparisons between[1]	CONC	TM	PM	ABM	SDF –T0	rSDF –T6
FRAC–1 unc *vs* RAW unc	–2.982 (0.002)	–2.197 (0.014)	–1.530 (0.063)	–1.492 (0.068)	–1.872 (0.031)	–1.158 (0.124)
RAW coll *vs* RAW unc	–3.059 (0.001)	–1.255 (0.105)	–2.040 (0.021)	–2.518 (0.006)	–2.790 (0.003)	–1.957 (0.025)
Fractionation vs *colloidal centrifugation of RAW semen*						
FRAC–1 unc *vs* RAW coll	–3.059 (0.001)	–0.863 (0.194)	–0.471 (0.341)	–1.102 (0.136)	–0.282 (0.389)	–1.377 (0.085)
Use of colloidal centrifugation on FRAC–1						
FRAC–1 coll *vs* FRAC–1 unc	–3.059 (0.001)	–0.760 (0.240)	–2.045 (0.021)	–1.805 (0.036)	–2.142 (0.016)	–2.703 (0.004)
FRAC–1 coll *vs* RAW coll	–2.668 (0.004)	–1.961 (0.025)	–0.942 (0.173)	–1.458 (0.073)	–1.832 (0.034)	–1.023 (0.153)

[1] unc: uncentrifuged; coll: after colloidal centrifugation.

tion differences were found to be statistically significant. The concentration of sperm in FRAC-1 was 2.5X greater than that corresponding to the colloidal centrifuged RAW ejaculate. Thirdly, we analysed the potential benefits of colloidal centrifugation performed on FRAC-1, so that uncentrifuged and colloidal centrifuged FRAC-1 samples were compared; although sperm concentration of FRAC-1 significantly decreased following colloidal centrifugation (Tables 1 and 2), there was significant improvement with respect to progressive motility, percentage of abnormal sperm, SDF-T0 and the rSDF-T6. Finally, we compared colloidal centrifuged FRAC-1 to the colloidal centrifuged RAW ejaculate and determined that the former possessed a sperm enriched fraction, as shown by a significantly higher sperm concentration, a significantly higher total motility and a lower SDF-T0; there was no improvement in progressive motility, abnormal morphology sperm or rSDF-T6 (Table 2).

Figure 1 is a summary of the improvement in sperm quality that was achieved by either direct colloidal centrifugation of RAW semen or colloidal centrifugation of FRAC-1. In both cases, we calculated, for each seminal characteristic, the percentage change detected relative to the observed values on RAW uncentrifuged samples. Sperm concentration was reduced by 45% after colloidal centrifugation but this deterioration in sperm number was significantly offset by ejaculate fractionation (p = 0.012). In addition, relative motion

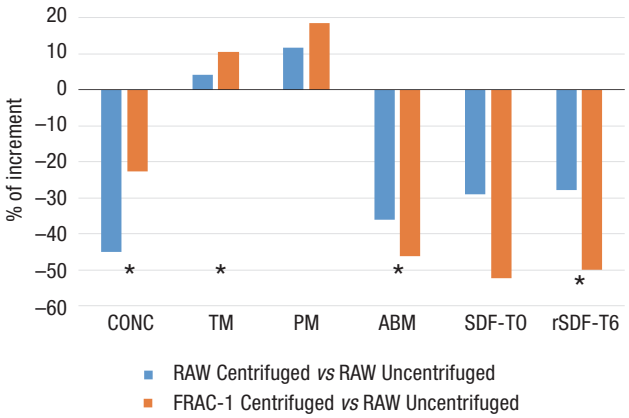

Figure 1. Comparison of the sperm improvement on seminal characteristics achieved after colloidal centrifugation and after colloidal centrifugation coupled with ejaculate fractioning. CONC: concentration (Z=–2.275; p=0.012), TM: total motility (Z=–1.961; p=0.025), PM: progressive motility (Z=–1.177; p=0.120), ABM: percentage of abnormal morphology sperm (Z=–2.080; p=0.019), SDF–T0: percentage of fragmented sperm at T0 (Z=–1.067; p=0.143), rSDF–T6: rate of sperm DNA fragmentation from T0 to T6 (Z=–1.647; p=0.050). Significant differences are marked by an asterisk (N=12 for all comparisons).

characteristics (total motility and progressive motility) were improved by colloidal centrifugation, but even more so when coupled with ejaculate fractionation (p = 0.025 for total motility). Similarly, the percentage of abnormal sperm, SDF-T0, and rSDF-T6 were reduced by colloidal centrifugation, but this reduction was further increased following ejaculate fractionation, though non-significantly in the case of SDF-T0 (p = 0.143).

Given the adverse effects of seminal plasma on stallion sperm preservation, application of colloidal centrifugation of sperm is gaining increasing popularity as a method of processing stallion semen (Morrell *et al*., 2010a; Mari *et al*., 2011; Morrell, 2011; Edmond *et al*., 2012). It has been described as an easy to use technique, requiring little equipment and consumables, although the cost per sample is currently the highest among the different sperm selection methods (Morrell, 2012). Our combined technique of utilising fractionated and colloidal centrifuged spermatozoa yielded significant improvements in all seminal characteristics, except for an expected loss of sperm concentration. While the positive effect of colloidal centrifugation on seminal characteristics of stallion sperm has also been demonstrated in previous studies (Johannisson *et al*., 2009; Morrell & Rodríguez-Martínez, 2009; Morrell *et al*., 2010b), we have demonstrated that this improvement can be further enhanced if the semen sample is fractionated on collection. Our observation of a reduction in rSDF-T6 following colloidal centrifugation is reported here for the first time in the stallion and is consistent with our other studies noting a possible association between slower DNA fragmentation dynamics and greater sperm viability in the female genital tract (López-Fernández *et al*., 2007; Cortés-Gutiérrez *et al*., 2008; Crespo *et al*., 2013).

In many species (*e.g.* human, equine, canine, porcine) the ejaculate is delivered in spurts or jets; this means that the collection of the whole naturally fractionated ejaculate into a single vial represents a "non-physiological" phenomenon, because such coalescence of the ejaculate fractions, whereby all the fluids mix together at a single time does not typically exist *in vivo*. Despite substantial research on characterization of the stallion ejaculate (Ackay *et al*., 2006; Kareskoski *et al*., 2006; 2010; 2011a,b; Kareskoski & Katila, 2008), there appears to have been less attention directed to the potential benefits and application of ejaculate fractionation in the equine artificial insemination industry. Stallion ejaculate fractions show a differential composition (Kareskoski *et al*., 2010; 2011a) because secretions from the accessory sex glands are released in a concerted sequential order (Magistrini *et al*., 2000). Secretions from the epididymis and ampulla have been associated with the sperm-rich fractions, whereas accessory gland

secretions predominantly contribute to the later fractions. Given this natural fractionation of the stallion ejaculate, we proposed that it might be possible to reduce the amount of seminal plasma without centrifugation. By simply using an open-end artificial vagina with the distal collecting cone removed (modified Polish or Krakow model, Tischner *et al.*, 1974), the sperm-rich fraction can be collected separately from the rest of the ejaculate, thus reducing the amount of seminal plasma and naturally increasing sperm concentration.

Edmond *et al.* (2012) drew attention to a downside for the use of colloidal centrifugation in stallions related to the characteristic large volume of stallion ejaculates, as compared to that of humans and most domestic animals. Morrell *et al*. (2009b) addressed this issue by increasing the size of the centrifuge tubes in order to accommodate a larger volume of semen per tube and, consequently, reducing the number of tubes needed for colloidal centrifugation of stallion ejaculates. However, as suggested by Edmond *et al.* (2012), this "scaling-up" imposes an economic constraint for most breeding studs, which are typically not equipped with the centrifugal rotors necessary for large tubes, and also because a large volume of the colloidal product is required per tube and to process the whole stallion ejaculate. Our results do not refute the potential benefit of colloidal centrifugation, but if ejaculate fractionation was additionally conducted, one would not be faced with the constraints of initial large ejaculate volume when performing colloidal centrifugation.

In conclusion, we found that stallion seminal characteristics could be improved by using colloidal centrifugation, but further enhanced, when coupled with ejaculate fractionation. This improved efficiency was observed with respect to progressive motility, abnormal sperm morphology, sperm DNA fragmentation and also sperm DNA longevity. While sperm concentration was reduced following colloidal centrifugation so that the number of sperm doses, will of course be lower, we predict that the resulting higher sperm quality will be associated with increased capacity for reproductive outcome.

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