Received: 3 January 2021 | Accepted: 9 March 2021

DOI: 10.1111/rda.13924

ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Variation among stallions in sperm quality after single layer centrifugation

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Funding information
Sveriges Lantbruksuniversitet, Grant/Award Number: NA; Ministry of Higher Education and Scientific Research, Iraq., Grant/Award Number: NA; Society for Reproduction and Fertility, Grant/Award Number: vacation scholarship

Abstract

Although single layer centrifugation (SLC) selects robust spermatozoa from stallion semen, the effect of individual variation has not been studied in detail. The objective of this study was to determine the variation among stallions in the effects of SLC on sperm quality during cooled storage for up to 48 hr. Semen samples from seven stallions (18 ejaculates) were split, with one portion being used for SLC and the other serving as a control (CON). Sperm quality (kinematics, reactive oxygen species (ROS) production, membrane integrity (MI) and chromatin integrity) were analysed at 0, 24 and 48 hr using computer-assisted sperm analysis and flow cytometry. Sperm quality was better in SLC than in CON at all timepoints, especially chromatin integrity and MI ($p < .0001$ for both), and some categories of ROS production (e.g. proportion of live hydrogen peroxide negative spermatozoa, $p < .0001$), but the degree of improvement varied among stallions and type of ROS ($p < .05$–$p < .0001$). Total and progressive motility were also better in SLC samples than in CON at 24 and 48 hr ($p < .0001$), although the effect on sperm kinematics varied. The interaction of treatment, time and stallion was not significant. In conclusion, sperm quality was better in SLC samples than in CON, although there was considerable individual variation among stallions. The improvement in sperm quality, particularly in chromatin integrity, was clearly beneficial, and therefore the use of this technique would be warranted for all stallion semen samples.

KEYWORDS
DNA fragmentation, equine semen, liquid stallion semen, semen evaluation, stored stallion sperm samples

1 | INTRODUCTION

Most equine inseminations are performed with cooled liquid semen up to 36 hr after collection (Aurich & Aurich, 2006). However, sperm quality varies considerably among stallions (Akbarinejad et al., 2020; Loomis & Graham, 2008), with concomitant variation in fertility (Colenbrander et al., 2003). Possible reasons for this high individual variation in sperm quality include inherent differences (Loomis & Graham, 2008), and the choice of sires for artificial insemination (AI) depending on the stallion's appearance or performance in sporting events rather than on sperm quality (Colenbrander et al., 2003).

Techniques for sperm quality analysis, such as Computer-Assisted Sperm Analysis (CASA) for motility and flow cytometry (FC), enable

sperm quality to be analysed in detail (Rodriguez-Martinez, 2013). Various in vitro sperm characteristics are associated with sperm fertility: membrane integrity (MI) is positively correlated with pregnancy rate after AI, whereas loss of chromatin integrity is negatively correlated (Morrell et al., 2009; Morrell, Stuhtmann, et al., 2014). Production of reactive oxygen species (ROS) as by-products of metabolism (Halliwell & Gutteridge, 2003) may also be useful as an indicator of potential fertility (Johannisson et al., 2014).

Colloid centrifugation, especially using a single layer of colloid (Single Layer Centrifugation, SLC), selects spermatozoa with intact membranes, good chromatin integrity (Morrell et al., 2010) and intact acrosomes (Costa et al., 2012) from the rest of the ejaculate. This improved sperm quality is retained during cooled storage, enabling the storage period prior to insemination to be increased (Costa et al., 2012; Lindahl et al., 2012), and was reflected in higher pregnancy rates following insemination (Morrell, Richter, et al., 2014). However, most previous studies investigated the effects of SLC over all stallions in the study, without examining differences among individuals. Therefore, the aim of the present study was to investigate whether the effect of SLC on stallion sperm quality varies among individuals.

2 | MATERIALS AND METHODS

2.1 | Animals and semen collection

Semen was collected from seven adult stallions (5–25 years old) at a commercial stud in Sweden during the breeding season. Three ejaculates were available from each of four stallions, and two ejaculates from the remaining three individuals ($n = 18$). A Missouri model artificial vagina was used after allowing the stallion to mount a phantom, as part of the stud's usual routine. The animals were housed and handled according to national and international regulations on animal care. No ethical approval was required for collection of semen from stallions using an artificial vagina in Sweden at the time of the study.

2.2 | Sperm concentration

Sperm concentration was measured immediately after collection using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) (Al-Kass et al., 2018). The ejaculate was extended in INRA 96 (IMV Technologies, l´Aigle. France) to give a final sperm concentration of 100×10^6 sperm/ml. The sample was split into two parts: control (CON) and SLC.

2.3 | Semen preparation

The SLC was carried out as described by Morrell et al. (2009). The extended semen (15 ml) was layered carefully over 15-ml Equicoll colloid in a 50-ml tube. After centrifugation at 300 *g* for 20 min, the supernatant and colloid was discarded, and the sperm pellet aspirated into a clean tube for resuspension in 5-ml extender. Aliquots were removed from all control and SLC samples at room temperature (23°C) for analysis (0 hr). All samples were then stored in the refrigerator at 5°C for 48 hr; samples were analysed at 24 and 48 hr after collection, equilibrating to room temperature before preparation each time.

2.4 | Computer-Assisted Sperm Analysis (CASA)

A SpermVision analyser (Minitüb GmbH, Tiefenbach, Germany), connected to an Olympus BX 51 microscope (Olympus, Tokyo, Japan), was used for motility evaluation on a daily basis, placing 5-μl semen sample (room temperature) on a warm slide (38°C), covered with an 18 × 18 mm coverslip. At least 1,000 spermatozoa were analysed in eight fields per slide for total motility (TM, %), progressive motility (PM, %), straightness (STR), straight line velocity (VSL, μm/s), velocity of the average path (VAP, μm/s), curvilinear velocity (VCL, μm/s), linearity (LIN), lateral head displacement (ALH, μm), wobble (WOB) and beat cross-frequency (BCF, Hz), assessed at 0, 24 and 48 hr. The settings for stallion spermatozoa were as follows: immotile spermatozoa VAP < 20; locally motile spermatozoa, VAP > 20 and <30, STR < 0.5, VCL < 9 (Al-Kass et al., 2018).

2.5 | Reactive Oxygen Species (ROS)

Sperm samples were stained with Hoechst 33258 (HO; Sigma, Stockholm, Sweden), which enters cells with damaged membranes, hydroethidine (HE; Invitrogen) to detect intracellular superoxide radical anion (Hossain et al., 2011), and 2′,7′dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen) to evaluate hydrogen peroxide (H_2O_2). The staining procedure used was as follows: two 300 μl aliquots at 2×10^6 sperm cells/ml were prepared with Cell Wash (Becton Dickinson, San José, CA, USA), and 9 μl of 40 μM HO, 9 μl of 40 μM HE as well as 9 μl of 2 μM DCFDA were added to each. In addition, 3 μl of 20 mM menadione (Sigma-Aldrich) were added to one of the samples to stimulate production of ROS. All samples were incubated for 30 min at 37°C, before analysis of 30,000 events using a FACSVerse flow cytometer (BD Biosciences, San José, CA, USA). Samples were excited with a (405 nm) violet laser and (488 nm) blue laser; a (527/32 nm) filter was used to detect green fluorescence, a (528/45 nm) filter was used to detect blue fluorescence, and a (700/54 nm) filter was used to detect red fluorescence. Samples were assessed at 0, 24 and 48 hr. Live cells were classified as hydrogen peroxide negative (live H_2O_2−), hydrogen peroxide positive (live H_2O_2+), superoxide negative (live SO−) and superoxide positive (live SO+).

2.6 | Membrane integrity

An aliquot (300 μl) from each sample was diluted with CellWash (Becton Dickinson, San José, CA, USA) to a final sperm concentration

of 2 × 10^6 spermatozoa/ml. The samples were stained with 0.6 μl of 0.02 mM SYBR14 and 3 μl of 12 μM propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA), and incubated for 10 min at 37°C. Samples were analysed by a FACSVerse flow cytometer (BD Biosciences). The forward and side scatter as well as green (527/32) and red (700/54) fluorescence were assessed at 0, 24 and 48 hr after semen collection. A total of 30,000 spermatozoa was evaluated. Spermatozoa with an intact membrane were stained with SYBR14 and spermatozoa with damaged membranes were stained with PI.

2.7 | Chromatin integrity

Samples were prepared for the Sperm Chromatin Structure Assay (Al-Kass et al., 2018) by adding an equal volume of buffer prepared from 0.01 M Tris-HCL, 0.15 M sodium chloride and 1 mM EDTA (50 μl each, 2 × 10^6 sperm/ml), before snap-freezing in liquid nitrogen. The samples were stored at −80°C until analysis.

Samples were thawed on ice and stained immediately with acridine orange, as follows: 90 μl of TNE buffer were added to 10 μl sample, followed by the addition of 200 μl of an acid-detergent solution, 0.17% Triton X-100 (Sigma); (0.15 M NaCl, and 0.08 M HCl; pH 1.2) to allow partial DNA denaturation; 30 s later, 600 μl of Acridine orange (AO, Sigma) staining solution (6 μg/ml in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl; pH 6.0) were added to the denatured suspension ofspermatozoa. The stained samples were analysed within 5 min using a FACSVerse flow cytometer (BD Biosciences). Ten thousand cells were evaluated, using a 527/32 bandpass filter for spermatozoa with intact DNA, which fluoresces green, while 700/54 bandpass filter was used to detect spermatozoa with single-stranded DNA which fluoresces red. The proportion of spermatozoa with damaged DNA was calculated from the ratio of red to (green + red) to give the DNA fragmentation index (%DFI) (Johannisson et al., 2009).

2.8 | Statistical analyses

All statistical analyses were performed with SAS software (SAS Institute Inc., 2013, version 9.4). Unless otherwise specified, data were analysed using the MIXED procedure for linear mixed models. The random effect of age and ejaculate nested within stallion was included in the model. A repeated effect of time (h after semen collection) was tested. The correlations between time and treatment were included by specifying a correlation structure AR (1) among residuals.

The residuals from the observations generated from the mixed models were tested for normal distribution. Data on Live SO+, Live H2O2+, Dead H2O2 + and Live SO + M, concentrations deviated from a normal distribution and were log-transformed. To improve clarity, avoid redundancy and facilitate interpretation, the respective log-transformed values are referred and presented as untransformed least square means values ± standard error of means (LSMeans ± sem) throughout.

The model included the fixed effect of stallion, time, treatment, the two-way interactions between time and treatment, stallion and time, stallion and treatment, and the three-way interaction between stallion, treatment and time. Estimated LSMeans from the models were adjusted using the Scheffé adjustment for multiple post-ANOVA comparisons and compared. Differences with $p \leq .05$ were considered significant.

3 | RESULTS

3.1 | Sperm kinematics

Total motility (Figure 1) and progressive motility (Figure 2) were higher for SLC than for controls at 24 and 48 hr for individual stallions ($p < .0001$). The mean total motilities over all stallions were as follows: at 0 hr: CON 83% ± 5.2, SLC 89% ± 5.2; N.S; 24 hr: CON 66% ± 5.2, SLC 91% ± 5.2 ($p < .0001$); 48 hr: CON 52% ± 5.2SLC 85% ± 5.2 ($p < .0001$). The mean progressive motilities for all stallions were as follows: 0 hr: CON 56% ± 4.6, SLC 62% ± 4.6; N.S; 24 hr: CON 38% ± 4.6, SLC 60% ± 4.6 ($p < .0001$); 48 hr: CON 26% ± 4.6SLC 55% ± 4.6 ($p < .0001$).

Progressive motility (Figure 2) varied among stallions ($p < .01$), with a greater improvement after SLC for some stallions than for others, for example stallion 1 where the difference in mean values

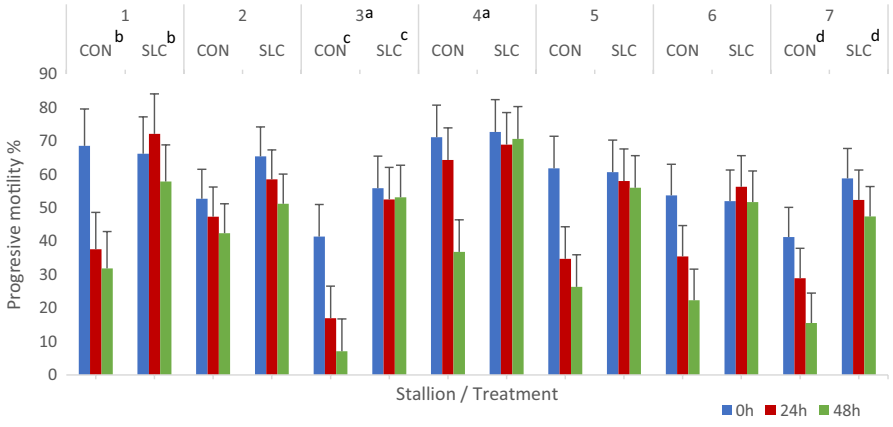

FIGURE 1 Total motility (Least Squares Means ± Standard Error) in ejaculates from seven stallions prepared by single layer centrifugation or control, evaluated at three time points ($n = 18$). Note: CON = control, SLC = Single Layer Centrifugation, stallions = 1 to 7, and storage time = 0, 24 and 48 hr. Similar superscript letters for CON and SLC indicate statistical difference between treatments within stallion, [a]$p < .05$, [b]$p < .001$

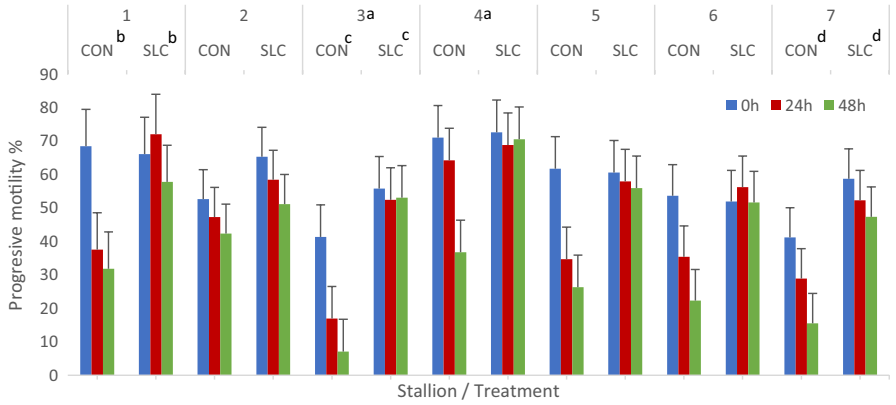

FIGURE 2 Progressive motility (Least Squares Means ± Standard Error) in ejaculates from seven stallions prepared by single layer centrifugation or control, evaluated at three time points ($n = 18$). Note: CON = control, SLC = Single Layer Centrifugation, stallions = 1 to 7, and storage time = 0, 24 and 48 hr. Similar superscript letters for stallion indicate significant differences between stallions; similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, $^{a,b}p < .05$, $^{c}p < .0001$, $^{d}p < .001$

was from 46% ± 8 in CON to 65% ± 8 in SLC ($p < .05$), stallion 3 where the difference in mean values was from 22% ± 6 in CON to 54% ± 7 in SLC ($p < .0001$), and stallion 7 where the difference in mean values was from 29% ± 6 in CON to 53% ± 6 in SLC ($p < .001$).

There was a stallion and treatment interaction for progressive motility ($p < .01$). There was a time and treatment interaction for total motility and for progressive motility ($p < .0001$). In addition, the interaction of stallion, time and treatment was significant for progressive motility ($p < .05$) but not for total motility (NS).

Sperm kinematics were mostly higher ($p < .01$) for CON than for SLC at 0 hr (Supplementary Table S1a–h), with the exception of BCF and the ratios STR, LIN and WOB. However, by 48 hr only WOB was higher for CON, CON 0.58 ± 0.01, SLC 0.53 ± 0.01 ($p < .01$), while STR and BCF were higher for SLC than for controls: for STR CON 0.69 ± 0.01, SLC 0.73 ± 0.01 ($p < .001$); BCF: CON 28 ± 0.6, SLC 31 ± 0.6 ($p < .01$). The other kinematics were not different between treatments.

There was a stallion and treatment interaction for BCF ($p < .05$), VCL ($p < .01$), VSL ($p < .01$), STR ($p < .01$) and WOB ($p < .01$), whereas the interaction was not significant for VAP, LIN and ALH (NS). There was a time and treatment interaction for ALH ($p < .01$), BCF ($p < .001$), VAP ($p < .0001$), VCL ($p < .0001$), VSL ($p < .0001$) and WOB ($p = .05$) but not for LIN or STR. The interaction of stallion, time and treatment was significant ($p < .05$) for progressive motility but was not significant for any sperm kinematics.

3.2 | Reactive oxygen species

The proportion of live hydrogen peroxide negative spermatozoa (live H_2O_2−; Table 1a) varied among stallions at all three time points ($p < .01$), whereas live H_2O_2 positive (H_2O_2+, Table 1b) was not significantly different. The proportion of live H_2O_2− spermatozoa was higher in SLC samples than CON for 6 of the 7 stallions. No stallion and treatment, time and treatment or stallion, time and treatment interaction was present for either live H_2O_2+ or live H_2O_2−. The stallions and time interaction was significant for live H_2O_2+ but not for live H_2O_2−.

Similarly, the proportion of live superoxide negative (SO−) spermatozoa was higher ($p < .0001$) in SLC samples than controls for all seven stallions at all three timepoints (Table 2a), and the proportion of live SO+ spermatozoa (Table 2b) was higher in SLC than CON for six stallions at all timepoints, albeit for different stallions.

There was a stallion and treatment interaction for live SO+ ($p < .01$), but no time and treatment interaction. The interaction of stallion, time and treatment was not significant for all ROS sub-populations.

Menadione addition stimulated superoxide production: the addition of menadione to semen samples resulted in reduced Live SO - and increased Live SO+ in both CON and SLC.

3.3 | Membrane integrity

MI (Figure 3) was higher for SLC than for controls at all timepoints ($p < .0001$). The mean values over all stallions were as follows: 0 hr: CON 74% ± 2.0, SLC 88% ± 2.0 ($p < .0001$); 24 hr: CON 77% ± 2.0, SLC 88% ± 2.0 ($p < .0001$); 48 hr: CON 68% ± 2.0 SLC 85% ± 2.0 ($p < .0001$).

There was a greater improvement in MI after SLC for some stallions than for others, for example stallion 1 where the mean difference was from 65% ± 3.6 in CON to 90% ± 3.6 in SLC ($p < .0001$), stallion 3 where the mean difference was from 63% ± 3.6 in CON to 79% ± 3.6 in SLC ($p < .001$), stallion 5 where the mean difference was from 75 ± 3.6 in CON to 92 ± 3.6 in SLC ($p < .01$) and in stallion 6 where the mean difference was from 74 ± 3.6% in CON to 91 ± 3.6 in SLC ($p < .01$).

There was a stallion and treatment interaction ($p < .01$), although there was no time and treatment interaction or stallion, time and treatment interaction.

TABLE 1 Hydrogen peroxide sub-populations (Least Squares Means ± Standard Error) for ejaculates from seven stallions prepared either by single layer centrifugation or control, evaluated at three time points ($n = 18$)

Stallions	Treatments	0 hr	24 hr	48 hr
(a) Live hydrogen peroxide negative spermatozoa (%)				
1	CON[d]	73 ± 10	63 ± 8	55 ± 8
	SLC[d]	89 ± 10	94 ± 8	89 ± 8
2	CON[g]	48 ± 8	52 ± 8	50 ± 8
	SLC[g]	83 ± 8	82 ± 8	80 ± 8
3[a]	CON[a]	57 ± 8	47 ± 8	42 ± 8
	SLC[a]	75 ± 8	73 ± 8	73 ± 8
4[a,b]	CON	84 ± 10	83 ± 8	83 ± 8
	SLC	107 ± 10	102 ± 8	97 ± 8
5	CON[c]	68 ± 8	56 ± 8	58 ± 8
	SLC[c]	86 ± 8	85 ± 8	78 ± 8
6	CON[g]	64 ± 8	54 ± 8	49 ± 8
	SLC[g]	88 ± 8	86 ± 8	84 ± 8
7[b]	CON[f]	57 ± 8	34 ± 8	47 ± 8
	SLC[f]	74 ± 8	74 ± 8	71 ± 8
Total	CON	65 ± 3[i]	56 ± 3[j]	55 ± 3[k]
	SLC	86 ± 3[i]	85 ± 3[j]	82 ± 3[k]
(b) Live hydrogen peroxide positive spermatozoa (%)				
1	CON	0.05 ± 3	0.01 ± 3	0.07 ± 3
	SLC	0.10 ± 3	0.04 ± 3	0.15 ± 3
2	CON	0.07 ± 3	0.1 ± 3	0.001 ± 3
	SLC	0.06 ± 3	0.08 ± 3	0.02 ± 3
3	CON	0.04 ± 3	0.04 ± 3	0.01 ± 3
	SLC	0.08 ± 3	0.04 ± 3	0.09 ± 3
4	CON	0.05 ± 3	0.03 ± 3	0.25 ± 3
	SLC	0.05 ± 3	0.17 ± 3	0.13 ± 3
5	CON	0.05 ± 3	0.02 ± 3	0.21 ± 3
	SLC	0.05 ± 3	0.13 ± 3	0.08 ± 3
6	CON	0.04 ± 3	0.02 ± 3	0.001 ± 3
	SLC	0.02 ± 3	0.29 ± 3	0.01 ± 3
7	CON	3.59 ± 3	22.71 ± 3	3.54 ± 3
	SLC	3.6 ± 3	3.7 ± 3	3.6 ± 3
Total	CON	0.05 ± 1.4	3.3 ± 1.3	0.55 ± 1.3
	SLC	0.55 ± 1.4	0.62 ± 1.3	0.57 ± 1.3

Note:: CON = control, SLC = Single Layer Centrifugation, stallions = 1 to 7, and storage time = 0 to 48 hr.

(a): Similar superscript letters for stallions 1–7 indicate significant differences between stallions; similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, and similar superscript letters for ROS values indicate significant differences within storage time, [a,b,c]$p < .05$, [d,e,f]$p < .01$, [g]$p < .001$ and [h,i,j,k]$p < .0001$.

3.4 | Chromatin integrity

The DNA fragmentation index (%DFI) was lower for SLC than for controls at all timepoints (Figure 4; $p < .0001$), 0 hr: CON 22% ± 1.4, SLC 9% ± 1.6 ($p < .0001$); 24 hr: CON 26% ± 1.4, SLC 10% ± 1.4 ($p < .0001$); 48 hr: CON 27% ± 1.4 SLC 10% ± 1.5 ($p < .0001$). Mean values for %DFI varied among stallions, from 9% ± 3 to 24% ± 3 ($p < .05$). There was a stallion and treatment interaction ($p < .05$), although no time and treatment interaction or stallion, time and treatment interaction.

4 | DISCUSSION

The study investigated the individual variation in stallion sperm quality after SLC selection. Sperm quality was improved in SLC

TABLE 2 Superoxide sub-populations (Least Squares Means ± Standard Error) for ejaculates from seven stallions prepared either by single layer centrifugation or control, evaluated at three time points ($n = 18$)

Stallions	Treatments	0 hr	24 hr	48 hr
(a) Live superoxide negative (%)				
1	CON	84.4 ± 10.3	70.3 ± 8.3	61.3 ± 8.3
	SLC	90.0 ± 10.3	93.5 ± 8.3	94.6 ± 8.3
2[e]	CON	53.2 ± 7.8	51.2 ± 7.8	50.1 ± 7.8
	SLC	78.9 ± 7.8	72.7 ± 7.8	60.9 ± 7.8
3[g]	CON [c]	50.7 ± 7.8	37.8 ± 7.8	28.1 ± 7.8
	SLC [c]	64.5 ± 7.8	61.5 ± 7.8	61.3 ± 7.8
4[a,b,d,e,g]	CON	101.3 ± 10.3	92.8 ± 8.3	90.1 ± 8.3
	SLC	121.5 ± 10.3	106.8 ± 8.3	99.4 ± 8.3
5[a]	CON	65.1 ± 7.8	52.9 ± 7.8	53.8 ± 7.8
	SLC	77.6 ± 7.8	71.0 ± 7.8	68.2 ± 7.8
6[b]	CON	65.6 ± 7.8	52.7 ± 7.8	47.5 ± 7.8
	SLC	84.9 ± 7.7	69.1 ± 7.8	67.2 ± 7.8
7[d]	CON	69.7 ± 7.8	60.2 ± 7.8	51.2 ± 7.8
	SLC	76.5 ± 7.8	77.6 ± 7.8	74.7 ± 7.8
Total	CON	70.0 ± 3.2[f]	59.7 ± 2.9[hr]	54.6 ± 3.0[i]
	SLC	84.8 ± 3.2[f]	78.9 ± 2.9[hr]	75.2 ± 2.9[i]
(b) Live superoxide positive (%)				
1	CON	0 ± 6.2	0 ± 5	0 ± 5.0
	SLC	7.9 ± 6.2	6.9 ± 5	7.3 ± 5.0
2	CON	0 ± 4.8	3.9 ± 4.8	2.7 ± 4.8
	SLC	7.3 ± 4.8	13.4 ± 4.8	21.9 ± 4.8
3	CON	5.5 ± 4.8	9.1 ± 4.8	13.2 ± 4.8
	SLC	9.2 ± 4.8	10.1 ± 4.8	10.7 ± 4.8
4	CON	0 ± 6.2	0 ± 5	1.6 ± 5
	SLC	0 ± 6.2	3.2 ± 5	4.6 ± 5
5	CON	5.4 ± 4.9	5.0 ± 4.9	5.5 ± 4.9
	SLC	9.7 ± 4.9	14.4 ± 4.9	12.1 ± 4.8
6	CON [b]	0 ± 5.2	2.8 ± 5.2	4.0 ± 5.2
	SLC [b]	4.4 ± 5.2	18.5 ± 5.2	20.5 ± 5.2
7	CON	3.7 ± 5.2	21.6 ± 5.2	10.5 ± 5.2
	SLC	11.6 ± 5.2	12.2 ± 5.2	10.9 ± 5.2
Total	CON	0.3 ± 2[d]	5.8 ± 1.9[c]	5.3 ± 1.9[a]
	SLC	6.4 ± 2[d]	11.2 ± 1.9[c]	12.2 ± 1.9[a]

Note:: CON = control, SLC = Single Layer Centrifugation, stallions = 1 to 7, and storage time = 0 to 48 hr.

(a): Similar superscript letters for stallion indicate significant differences between stallions; similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, and similar superscript letters for superoxide values indicate significant differences within storage time, [a,b,c,d]$p < .05$, [e,f]$p < 0.01$, [g]$p < .001$ and [h,i]$p < .0001$.

(b): Similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, and similar superscript letters for superoxide values indicate significant differences within storage time, [a]$p < .05$, [b,c]$p < .01$, [d]$p < .0001$.

samples compared to CON, in agreement with previous studies (Al-Kass, 2019; Costa et al., 2012; Johannisson et al., 2009; Pessoa et al., 2020), and there was a stallion*treatment interaction for most parameters except some kinematics and live H_2O_2 categories.

An improvement in CASA sperm motility was observed in SLC-selected samples, in agreement with previous studies (Al-Kass et al., 2018; Costa et al., 2012; Johannisson et al., 2009; Morrell, Dalin, et al., 2009; Morrell, Johannisson, et al., 2009) and also for sperm kinematics (Al-Kass et al., 2018; Guimaraes et al., 2015). In

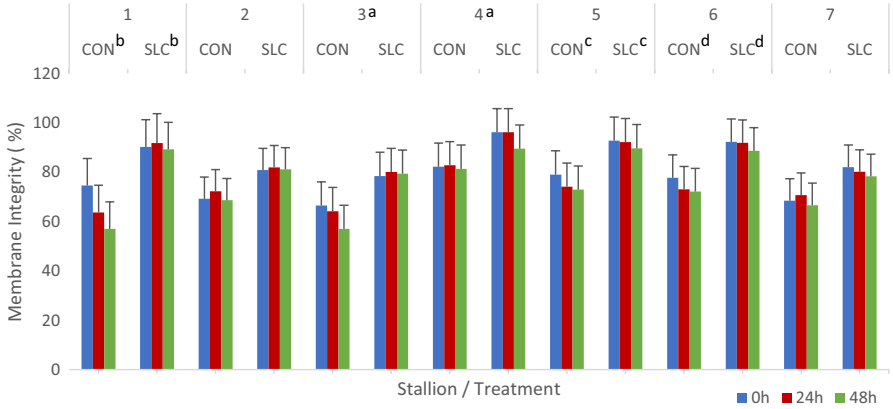

FIGURE 3 Proportion of spermatozoa with intact membranes (Least Squares Means ± Standard Error) in ejaculates from seven stallions prepared by single layer centrifugation or control, evaluated at three time points ($n = 18$). Note: CON = control, SLC = Single Layer Centrifugation, stallions = 1 to 7, and storage time = 0 to 48 hr. Similar superscript letters for stallion indicate significant differences between stallions; similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, $^{a}p < .05$, $^{b}p < .0001$, $^{c,d}p < .01$

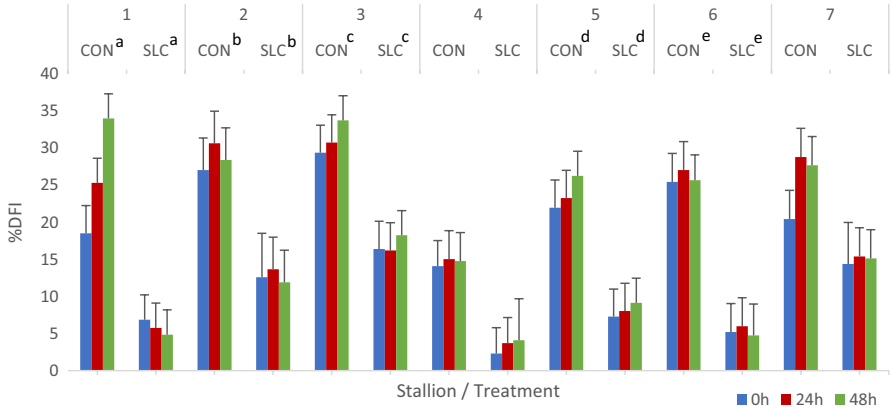

FIGURE 4 Proportion of spermatozoa with fragmented chromatin (Least Squares Means ± Standard Error) in ejaculates from seven stallions prepared by single layer centrifugation or control, evaluated at three time points ($n = 18$). Note: CON = control, SLC = Single Layer Centrifugation, stallions 1 to 7, and storage time = 0, 24 and 48 hr. Similar superscript letters for stallion indicate significant differences between stallions; similar superscript letters for CON and SLC indicate significant differences between treatments within stallion, $^{a,e}p < .0001$, $^{b,c}p < .01$, $^{d}p < .001$

contrast, Al-Essawe et al. (2018) indicated that sperm kinematics in frozen-thawed sperm samples were not different between SLC and CON.

The MI was increased after SLC in the present study, in agreement with Pessoa et al. (2020), Al-Kass et al. (2018), Morrell, Johannisson, et al. (2009), and Johannisson et al. (2009). The MI was previously shown to be positively correlated with pregnancy rate in mares inseminated with conventional sperm doses (Morrell et al., 2008). Therefore, the increased MI in SLC samples could explain the increased fertility seen in an AI trial where SLC sperm doses were compared with controls (Morrell, Richter, et al., 2014).

In the present study, the %DFI was decreased after SLC (5 out of 7 stallions), in agreement with previous studies (Al-Essawe et al., 2018; Al-Kass et al., 2018; Crespo et al., 2013; Johannisson et al., 2009; Morrell, Dalin, et al., 2009; Morrell, Johannisson, et al., 2009), although Costa et al. (2012) did not observe an effect of SLC on chromatin integrity. Since %DFI was reported to be negatively correlated with pregnancy rate (Morrell et al., 2008), a decreased %DFI could have contributed to the increased fertility seen in SLC samples in an AI trial (Morrell, Richter, et al., 2014).

Most previous studies showed that the length of time for which sperm quality was retained during storage was prolonged in SLC samples compared to controls (Al-Kass et al., 2018; Johannisson et al., 2009; Morrell, Johannisson, et al., 2009). The results of the present study are in accordance with these observations. The effect, however, varied among stallions and differed for different parameters. Thus, stallions 1 and 3 showed improvements in total motility, progressive motility, live H_2O_2−, chromatin integrity and MI after SLC, whereas for stallions 5 and 6, the improvements included live H_2O_2−, chromatin integrity and MI. For stallion 7, the SLC samples showed improved progressive motility and live H_2O_2-, whereas for stallion 2, the SLC samples showed improved live H_2O_2− and chromatin integrity. The remaining stallion (stallion 4) did not show any marked improvements, mainly because the sperm quality was very

good in CON anyway. However, even for this stallion, there were some benefits from SLC in terms of improved sperm quality by 48 hr.

Our results indicate high individual variation in semen quality among stallions, in agreement with Akbarinejad et al., (2020). Most stallions showed a marked improvement in sperm quality for SLC compared to CON. Therefore, it is recommended to test each individual stallion at the start of the breeding season to see if sperm quality can be improved by SLC or not. However, if it is not considered practical to test all stallions, or if it is desired to have the same semen preparation protocol for all ejaculates, it would be advisable to use SLC on all sperm samples. In this way, all insemination doses would be of high quality, especially with regard to chromatin integrity.

Although fertilizing ability was not tested in the present study, it can be inferred from the results for MI and chromatin integrity. The higher proportion of H_2O_2 negative spermatozoa in the SLC samples may have contributed to the improved longevity, as reported previously (Al-Essawe et al., 2018; Morrell et al., 2016). In previous studies, the proportion of live H_2O_2 negative spermatozoa was correlated with progressive motility (Johannisson et al., 2014), and MI (Akbarinejad et al., 2020). There was less deterioration over time in the SLC samples compared with CON for most parameters analysed. In a previous study, the storage time for SLC samples could be increased to 96 hr without compromising sperm fertility (Lindahl et al., 2012). This increased storage time would be of considerable benefit when planning inseminations, since it is not always possible to predict when the mare will ovulate, or if transport of the insemination doses could be prolonged.

5 | CONCLUSION

The improvement in stallion sperm quality following SLC was seen for many aspects of sperm quality although the extent varied among individuals. This has important implications for AI centres when choosing stallions or ejaculates for AI and is particularly important if transport times for insemination doses could be prolonged. It is recommended to test ejaculates at the beginning of the breeding season to determine which samples are likely to benefit from this semen-processing technique.

ACKNOWLEDGMENTS

The authors are grateful to the Swedish University of Agricultural Sciences and the University of Mosul/College of veterinary medicine for providing the facilities and support, and to the Society for Reproduction and Fertility for funding the project via a vacation study grant awarded to Mr A Brown. ZAK was sponsored by the Ministry of Higher Education and Scientific Research, Iraq. The other authors were supported by the Swedish University of Agricultural Sciences.

CONFLICT OF INTEREST

JMM is the inventor and one of the patent holders of the colloid formulation used in this study.

AUTHORS CONTRIBUTION

ZA-K helped to collect and analyse data, and wrote the original draft of the paper; AB collected most of the data; AJ supervised the flow cytometric analysis of samples and helped design the study; TN did the statistical analysis; JMM designed the study, provided the funding, supplied the colloid and edited the draft manuscript. All authors read and approved the final draft.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Al-Kass Z, Brown A, Johannisson A, Ntallaris T, Morrell JM. Variation among stallions in sperm quality after single layer centrifugation. *Reprod Dom Anim*. 2021;56:848–856. https://doi.org/10.1111/rda.13924