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Original Research

Stored Stallion Sperm Quality Depends on Sperm Preparation Method in INRA82 or INRA96

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ABSTRACT

Removal of seminal plasma facilitates stallion sperm survival during storage, but washing may damage sperm chromatin. Therefore, sperm quality was compared in samples following single-layer centrifugation (SLC) or sperm washing and controls (extension only) in two extenders, INRA82 and INRA96. Ejaculates from six stallions were split among six treatments: SLC, sperm washing, and controls, in INRA82 and INRA96. Sperm motility and acrosome status were evaluated at 0, 24, 48, 72 and 96 hours; morphology at 0, 24, 48, 72 hours and chromatin integrity at 0 and 96 hours, with storage at 6°C. Sperm samples in INRA96 had better motility, acrosome status, and normal morphology than samples in INRA82. The SLC samples had higher motility and fewer reacted acrosomes than controls, and lower fragmented chromatin than washed samples. Fewer spermatozoa with tail defects were observed after SLC than after sperm washing; spermatozoa washed in INRA82 had fewer tail defects than those washed in INRA96. In conclusion, sperm quality (except for morphology) was better in INRA96 than in INRA82 and was better in SLC samples than in washed samples or controls. The SLC method is a useful adjunct to stallion sperm preparation, especially for storage before artificial insemination.

1. Introduction

Most equine artificial inseminations (AI) are now performed with cooled semen rather than with fresh semen immediately after collection [1]. Removing most of the seminal plasma (SP) from stallion ejaculates could have a beneficial effect on stabilizing sperm membranes during cooled storage [2] and might reduce chromatin damage [3], presumably by removing sources of reactive oxygen species. Furthermore, it may reduce the inflammatory response in mares prone to post-breeding endometritis—an exaggerated form of the normal inflammatory response in the uterus [4,5]. Therefore, in some countries, for example Germany, Austria [6], and The Netherlands [7], it is standard practice to remove SP by centrifugation (sperm washing) when preparing cooled semen doses for AI. However, sperm washing does not increase the pregnancy rates following AI compared to non-washed insemination doses [2] and is not carried out routinely in countries such as the UK [8], France [9], and Italy [10] because it may have a detrimental effect on sperm quality, such as damaging sperm chromatin. If sperm concentration is low, centrifugation of the extended ejaculate may be carried out to allow subsequent removal of most of SP, with resuspension of the pellet in a small quantity of fresh semen extender. This procedure has the effect of allowing the sperm concentration in the sample to be increased for insemination.

Colloid centrifugation of semen might be an alternative to sperm washing, with the additional benefit that it selects the spermatozoa that are morphologically normal, viable and have good chromatin integrity [11]. The colloid also removes SP proteins coating the surface of spermatozoa [12]. Stallion sperm samples prepared by colloid centrifugation were still fertile after storage at 6°C for 96 hours [13]. Thus, this technique could offer a viable alternative to sperm washing for preparing stallion semen for AI. Although some studies

Animal welfare/ethical statement: Animals were housed and handled according to national and institutional regulations for animal care and use.

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

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have been carried out to compare either sperm washing [2] or single-layer centrifugation (SLC) [11] with controls, there have been few detailed studies to compare both sperm washing and SLC with controls, with sperm quality in the resulting samples being analyzed during storage for several days [14].

Traditionally, stallion semen extenders were based on skimmed milk, for example Kenney's extender, INRA82, but this ingredient may introduce variability [9]. Moreover, some components of milk may be toxic to spermatozoa [15]. Therefore, commercial extenders have been developed that are chemically defined, based on the phosphocaseinate fraction of milk, for example INRA96 [9]. It was reported that the storage time of stallion semen at 15°C could be increased beyond the usual 24–36 hours using INRA96, although fertility was reduced in comparison to immediate insemination [9].

The objective of the present study, therefore, was to compare the effect of SLC, sperm washing, and extension alone on sperm kinematics, acrosome integrity, sperm morphology, and sperm chromatin integrity, in sperm samples stored at 6°C for 96 hours using two extenders, INRA82 and INRA96.

2. Materials and Methods

2.1. Experimental Design

Four ejaculates were collected from each of six stallions. Each ejaculate was split among the following treatments: (1) SLC in INRA82 (A1); (2) SLC in INRA96 (A2); (3) sperm washing in INRA82 (centrifugation at 850 × g for 10 minutes; resuspension of sperm pellet to a concentration of 30 × 10^6/mL) (B1); (4) sperm washing in INRA96 (B2); (5) extension (to a sperm concentration of 30 × 10^6/mL) in INRA82 (C1), (6) extension in INRA96 (C2). Thus C1 and C2 were controls. The sperm samples were stored at 6°C and evaluated as follows: sperm motility and acrosome status were evaluated at 0, 24, 48, 72, and 96 hours, and additional aliquots were taken at 0 and 96 hours for subsequent analysis of chromatin integrity. Samples for morphology evaluation were taken from two ejaculates per stallion at three time points and from the native ejaculate at time 0 hours.

2.2. Animals and Semen Collection

Semen was collected regularly from six Hanoverian stallions of known fertility, maintained at the National Stud of Lower Saxony, Celle, Germany, as part of the normal stud routine. The stallions, aged 5–19 years, were housed in stalls on straw bedding or shavings, fed oats and hay three times daily, with water available *ad libitum,* according to national and institutional regulations for animal care and use.

After allowing the stallion to mount a phantom, semen was collected using an artificial vagina (Hanover model; Minitüb; Tiefenbach, Germany). The ejaculate was filtered through gauze to remove gel. Sperm concentration was measured using a Nucleocounter SP-100 (ChemoMetic A/S, Allerød, Denmark) and was adjusted to the desired concentration with warm semen extender. The extenders were either skimmed milk extender (INRA82) made by mixing equal volumes of ultra-heat treated skimmed milk and glucose saline solution (25 g glucose monohydrate, 1.5 g raffinose pentahydrate, 0.25 g tri-sodium citrate dihydrate, 0.41 g potassium citrate monohydrate, 4.76 g HEPES, 0.5 g penicillin, 0.5 g gentamycin; pH 6.8–7.2, osmolarity 300–330m Osmkg-1), or commercially available INRA96 (IMV Technologies, l'Àigle, France).

2.3. Single-Layer Centrifugation

Extended semen (15 mL) was carefully layered over 15 mL Androcoll-E (supplied by the senior author, JMM) in a 50 mL tube [16]. After centrifuging at 300 × g for 20 minutes, the supernatant consisting of extender and SP was removed, followed by careful removal of the colloid down to the last 2 mL. The sperm pellet was aspirated from below the remaining colloid and was resuspended in the appropriate extender to a sperm concentration of 30 × 10^6/mL.

2.4. Sperm Morphology

An aliquot (100 μL) of each sperm sample was stained with 300 μL nigrosin-eosin solution: 10% nigrosin, 0.7% eosin, 3.75 mM glucose, 1.88 mM KH_2PO_4, 3.75 mM Na_2HPO_4, 5.78 mM NaK tartrate [17]. Air-dried smears were prepared for evaluation of 200 spermatozoa per sample, using 10 × 100 magnification with oil immersion [18]. Note that samples for morphology were only taken for two ejaculates per stallion, sampling times were 0, 24, 48 and 72 hours. If a spermatozoon had more than one defect, only the more serious defect was counted.

2.5. Computer-Assisted Sperm Motility Analysis

Sperm motility was assessed by computer-assisted sperm analysis (CASA) using SpermVision (Minitüb; Tiefenbach, Germany) connected to a microscope with a temperature-controlled stage (37°C) and a camera (60 frames/s) for recording motility characteristics. An aliquot (500 μL) of each sample was incubated for 5 minutes, after which 3 μL was inserted into a 20 μL chamber (Leja Products BV, Nieuw Vennep, The Netherlands). Kinematics were calculated as mean values from eight microscopic fields (at least 1,000 spermatozoa). The following kinematics were recorded: total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL μm/s), straight line velocity (VSL μm/s), average path velocity (VAP μm/s), amplitude of lateral head deviation (ALH; μm), and beat cross-frequency (BCF; Hz). Spermatozoa were considered progressively motile when straightness (VSL/VAP) was > 0.5 and VAP was > 40 μm/second.

2.6. Plasma and Acrosomal Membrane Integrity

Plasma and acrosomal membranes were evaluated simultaneously. An aliquot (5 μL) of each sperm sample containing 50 × 10^6 cells/mL was mixed with 495 μL modified Tyrodes medium (100 mM NaCl, 3.1 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 21.6 mM Na-lactate, 1 mM Na-pyruvate, 10 mM HEPES, pH 7.5, 3 g/L bSA), to which 3 μM PI (Sigma-Aldrich, St. Louis, MO) and 0.45 μM FITC-PNA (Vector Laboratories, Burlingame, CA) were added. The mixture was incubated for 10 minutes at 37°C in 5% CO_2, before analysis using a Quanta flow cytometer (Beckman–Coulter, Fullerton, CA) equipped with a 488 nm argon ion laser of 22 mW for excitation, with band pass 525/30 nm and long-pass 670 nm filters for detecting green and red fluorescence, respectively. At least 5,000 spermatozoa, selected according to their side scatter and electronic volume properties, were evaluated per sample. Spermatozoa that were both PI- and FITC-PNA-negative were considered viable with intact plasma and acrosomal membranes. For the purposes of this study, only results for membrane-intact (live) spermatozoa (acrosome intact and acrosome reacted) are shown.

2.7. Sperm Chromatin Structure Assay

The method of Evenson et al [19] was used. Samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis. The thawed samples were diluted in TNE buffer (0.15 M NaCl, 0.01 M TRIS-HCl, 1 mM disodium EDTA, pH 7.4) at approximately 2 × 10^6 cells mL/mL. From this solution, 200 μL was diluted with 400 μL acid solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) for 30 seconds before adding 1.2 mL acridine orange (Polysciences,

Table 1
Effect of sperm processing treatments (sperm washing or single-layer centrifugation), and effect of extender (INRA82 or INRA96) on total at several time points during cooled storage (n = 24).

Time	Motility	A1	A2	B1	B2	C1	C2
0 h	TM %	82 ± 6^{ab}	80 ± 6	75 ± 8^{a}	74 ± 9	75 ± 7^{b}	74 ± 9
24 h	TM %	80 ± 18^{ad}	80 ± 7^{b}	71 ± 10^{a}	75 ± 8	68 ± 10^{d}	73 ± 10^{b}
48 h	TM %	75 ± 9^{fg}	$77 \pm 7e$	61 ± 16^{fgh}	74 ± 10	46 ± 23^{gh}	67 ± 13^{e}
72 h	TM %	63 ± 18^{dgh}	74 ± 10^{di}	42 ± 23^{gj}	67 ± 11^{k}	29 ± 20^{hj}	52 ± 20^{ik}
96 h	TM %	53 ± 28^{dgh}	64 ± 21^{gi}	34 ± 25^{dj}	64 ± 17^{tk}	19 ± 18^{hj}	46 ± 24^{ik}

Abbreviations: AI, SLC with INRA82; A2, SLC with INRA96; B1, sperm washing with INRA82; B2, sperm washing with INRA96; C1, extended in INRA 82; C2, extended in INRA96; TM, total motility; PM, progressive motility.
Same superscripts within a row indicate significance: [a,b,c], $P < .05$; [d,e,f], $P < .01$; [g,h,i,j,k,l], $P < .001$.

Warrington, PA) staining solution (0.15 M NaCl, 0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M disodium EDTA, pH 6.0, containing 6 μg/mL acridine orange). Samples were incubated on ice for 3 minutes, after which 10,000 cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany) equipped with a 488 nm argon ion laser of 15 mW for excitation and band pass filters 530/30, 582/42 nm and a long pass 650 nm filter for detecting green, orange, and red fluorescence, respectively. Acridine orange stains normal double-stranded DNA green, and denatured single-stranded DNA red; the acid treatment potentially denatures damaged DNA. The DNA fragmentation index (%DFI) was calculated from the fractions of cells with single- and double-stranded DNA.

2.8. Statistical Analyses

Analysis of variance (ANOVA) was performed using the Statistical Analysis System software (SAS Institute, V.9.4, Cary, NC). The statistical model included the fixed effects of treatment (6 classes), time (5 classes), and the interaction between time and treatment. The model also included the random effect of ejaculate, nested within stallion. For sperm morphology, two variables (% total necks and % of cytoplasmic drops), with a distribution deviating from normality, were log transformed before the analyses.

All results are expressed as least-square means value ± standard error and P values < .05 were considered statistically significant. Scheffe adjustment for multiple-post ANOVA comparisons was used.

3. Results

The descriptions in the text reflect trends because of the multitude and complexity of the data. For the P values of specific comparisons, please see the appropriate tables and figures.

3.1. Computer-Assisted Sperm Analysis Assessment

Total motility (Table 1) and progressive motility (Fig. 1) were higher in INRA96 than INRA82 for each treatment. Sperm motility was also higher if processed by SLC than by sperm washing or extension, except for sperm washing in INRA96 at 0 and 96 hours.

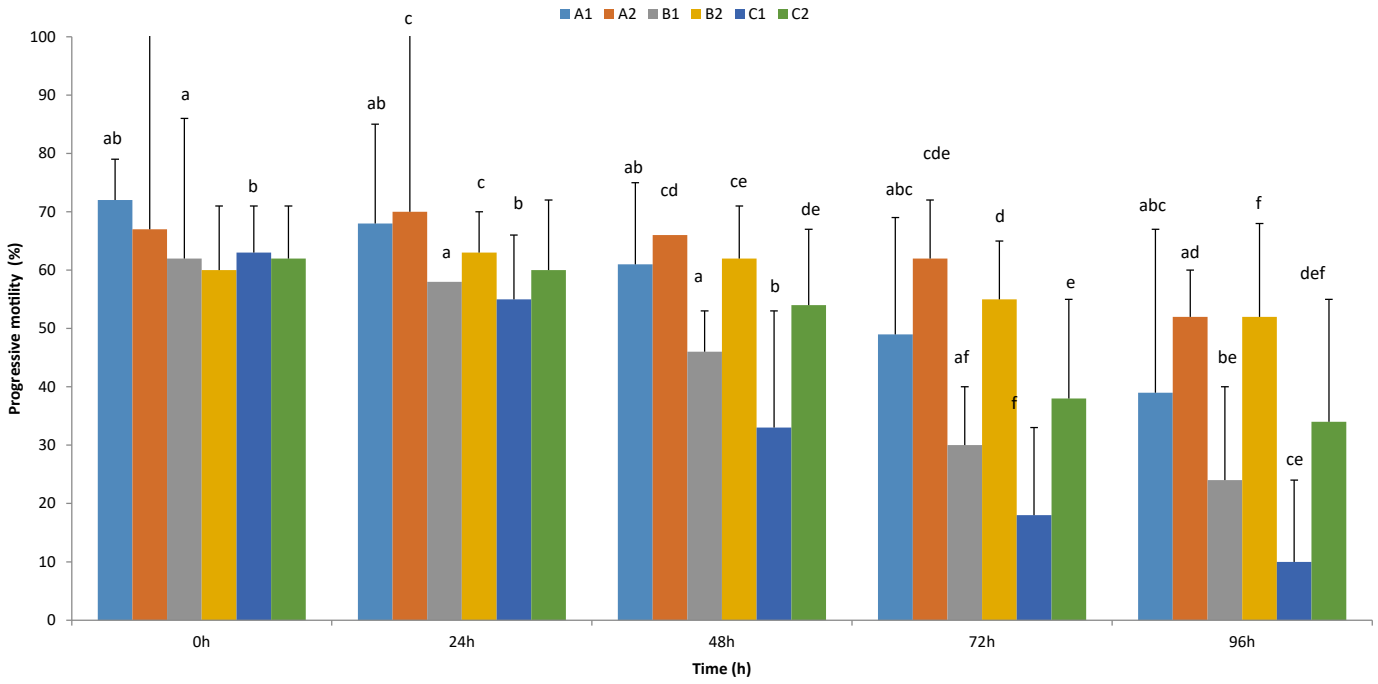

Fig. 1. Effect of sperm handling method (single-layer centrifugation, sperm washing, or extension only) and extender (INRA82 or INRA96) on mean (±SD) progressive motility for single-layer centrifugation, sperm washing, and extension treatments during cooled storage for 96 hours (n = 24). A1, single-layer centrifugation in INRA82; A2, single-layer centrifugation in INRA96; B1, sperm washing in INRA82; B2, sperm washing in INRA96; C1, extension in INRA82; C2, extension in INRA96. The following differences were statistically significant: 0 hours: a, b $P < .01$; 24 hours: a $P < .01$, b $P < .001$, c $P < .05$; 48 hours a,b,c,d $P < .001$, e $P < .05$; 72 hours. 72 hours: a,b,c,e,f, $P < .001$, d $P < .05$; 96 hours: a,b,c,d,e,f $P < .001$.

Table 2
Effect of sperm processing treatments (sperm washing or single-layer centrifugation) and effect of extender (INRA82 or INRA96) on sperm velocity at several time points during cooled storage (n = 24).

Time	Kinematic	A1	A2	B1	B2	C1	C2
0 h	VCL	142 ± 11adeg	131 ± 12af	158 ± 9dei	135 ± 13Fih	164 ± 10g	155 ± 16fh
	VSL	50 ± 4 ad	47 ± 5 ab	49 ± 3 ce	45 ± 10 beg	53 ± 4 cd	50 ± 4 bg
	VAP	71 ± 6 dgh	65 ± 6 di	79 ± 3 bgjj	70 ± 7 ajk	84 ± 6 beh	78 ± 8 eik
24 h	VCL	146 ± 32 ag	143 ± 14 h	157 ± 10 ab	147 ± 9 d	168 ± 15 bg	163 ± 14 dh
	VSL	50 ± 11	49 ± 4	48 ± 3	47 ± 3 d	81 ± 7	81 ± 8 d
	VAP	72 ± 16 dg	71 ± 6 h	77 ± 4 abd	77 ± 5 ac	81 ± 7 bg	81 ± 8 ch
48 h	VCL	142 ± 15 a	144 ± 17 g	144 ± 15 b	152 ± 12 h	154 ± 24 ab	174 ± 14 gh
	VSL	68 ± 10 ad	68 ± 10	73 ± 7 dg	77 ± 6 g	74 ± 10 ab	83 ± 7 b
	VAP	68 ± 10 ae	70 ± 8 g	72 ± 7 abd	77 ± 6 bdf	74 ± 10 eh	83 ± 7 fgh
72 h	VCL	138 ± 17 g	142 ± 16 h	135 ± 20 a	147 ± 16 ad	137 ± 29 Gi	160 ± 27 dhi
	VSL	65 ± 11 dgh	69 ± 8 ai	67 ± 8 dgkj	74 ± 6 ijl	68 ± 9 Ehk	78 ± 9 eal
	VAP	65 ± 11	69 ± 8 dg	67 ± 8 h	74 ± 6 d	68 ± 9 i	73 ± 9 ghi
96 h	VCL	130 ± 28 D	136 ± 19 G	131 ± 25 Ah	142 ± 15 Ab	114 ± 46 Dhi	154 ± 24 bgi
	VSL	63 ± 12 Dg	65 ± 9 E	66 ± 10Adf	73 ± 8 A	59 ± 16 Fgh	76 ± 9 eh
	VAP	63 ± 12 A	65 ± 9 Gh	66 ± 10 Di	73 ± 8 Dg	59 ± 16 Aij	76 ± 9 hj

Abbreviations: VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.
Same letters within a row denote significant differences: abc $P < .05$, def $P < .01$, ghijk $P < .001$.

There was a significant interaction between treatment and time, and also with the type of extender. Mean values of sperm kinematics also showed significant effects of treatment, extender, and time, and a significant interaction between treatment, extender, and time. Velocity (VCL, VSL, and VAP) tended to be higher in INRA82 than INRA96 at most time points (Table 2); VCL was highest for extended semen and lowest for SLC (A < B < C). The ALH was higher in extended semen than in SLC or washed samples (C > B = A) and was also higher in INRA82 than in INRA96 initially, but these differences disappeared with time in storage (Table 3). The BCF decreased with time in the extended samples without SLC, especially where INRA82 was used (Table 3). The effect of extender alone was not different.

3.2. Acrosome Status

Note that only results for live acrosome intact spermatozoa are considered here. There was a significant effect of treatment, time, and extender on the proportion of live acrosome intact spermatozoa (Fig. 2). The proportion of live spermatozoa with unreacted acrosomes was maintained in the SLC groups over 96 hours, regardless of extender. For the control and sperm washing groups, higher proportions of live spermatozoa with intact acrosomes were found in INRA96 than INRA82, from 24 hours onward.

3.3. Morphology

The proportion of abnormal spermatozoa in the different groups at different time points is shown in Table 4. The most common abnormalities were head defects (70% of all abnormalities) followed by tail defects (12%) and detached heads (11%). There was no effect of storage time on head defects (Table 5) or tail defects (Table 6) for the differetn treatments.

There were fewer tail defects in the SLC samples compared to the washed samples (A1 vs. B1, $P < .01$; and A2 vs. B2, $P < .05$). Sperm samples that were washed in INRA82 had fewer tail defects than those washed in INRA96 ($P < .05$). There was a significant time*treatment interaction, with an increase in abnormal spermatozoa after 48 hours compared to 0 and 24 hours.

3.4. Chromatin Integrity

Treatment had a significant effect on %DFI (Fig. 3) ($P < .001$), with the most chromatin damage being seen in the sperm washing group. The least chromatin damage was observed in the SLC group, regardless of extender (A1 vs. B1 and C1; A2 vs. B2 and C2, $P < .001$; A1 vs. C1, $P < .05$). There were differences among stallions ($P < .01$).

4. Discussion

In this experiment, three sperm processing treatments (control, i.e., extension only, sperm washing and SLC) were compared for their effect on sperm kinematics, proportion of live unreacted acrosomes, morphology, and chromatin integrity over time during cooled storage. There was a significant interaction between extender, treatment, and time: sperm suspensions showed higher motility and acrosome integrity in INRA96 than in INRA82. Sperm washing and SLC resulted in better quality samples than controls. In

Table 3
Effect of sperm processing treatments (sperm washing or single-layer centrifugation) and extender (INRA82 or INRA96) on ALH and BCF at several time points during cooled storage (n = 24).

Time	Kine-matics	A1	A2	B1	B2	C1	C2
0 h	ALH	3.6 ± 0.3 Dgh	3.3 ± 0.3 Dij	4.2 ± 0.2 Gk	3.6 ± 1.8 Ikl	4.2 ± 0.2 H	4.0 ± 0.4 jl
	BCF	31.7 ± 2.2 D	31.3 ± 2.6 G	29.3 ± 1.1 D	28.4 ± 1.3 Eg	32.8 ± 2.6	31.0 ± 2.7 e
24 h	ALH	3.8 ± 0.8 Gh	3.6 ± 0.3 Aij	4.2 ± 0.2 Dg	3.9 ± 0.4 Adei	4.4 ± 0.3 H	4.3 ± 0.4 ej
	BCF	31.6 ± .6.8 G	31.9 ± 3.6 H	29.2 ± 1.1 G	28.5 ± 1.4 Dh	30.7 ± 1.7	30.8 ± 2.5 d
48 h	ALH	3.6 ± 0.4 Gh	3.6 ± 0.4 Ij	4.0 ± 0.4 Ag	4.1 ± 0.4 Ik	4.3 ± 0.4 Abh	4.5 ± 0.3 bjk
	BCF	32.5 ± 3.1 Gh	32.8 ± 4.3 Ij	28.4 ± 1.6 G	29.1 ± 1.8 I	28.4 ± 3.0 H	29.9 ± 2.3 J
72 h	ALH	3.6 ± 0.4 gh	3.7 ± 0.4 Ij	4.0 ± 0.4 G	4.1 ± 0.5 Il	4.3 ± 0.4 Adh	4.6 ± 0.3 ajl
	BCF	32.2 ± 2.8 Gh	32.4 ± 3.6 I	27.5 ± 2.7 g	28.4 ± 1.4	26.6 ± 4.5 H	28.0 ± 3.2 i
96 h	ALH	3.6 ± 0.5 Def	3.6 ± 0.3 Dgh	4.0 ± 0.4 e	4.0 ± 0.5 gi	4.0 ± 1.1 fj	4.4 ± 0.4 hij
	BCF	30.7 ± 4.1 gh	30.7 ± 3.1 ai	25.6 ± 4.3 bgj	28.1 ± 1.0 ab	12.8 ± 8.4 hjk	26.6 ± 3.4 ik

Similar superscripts within a row denote significant differences: ab $P < .05$, de $P < .01$, ghijkl $P < .001$.

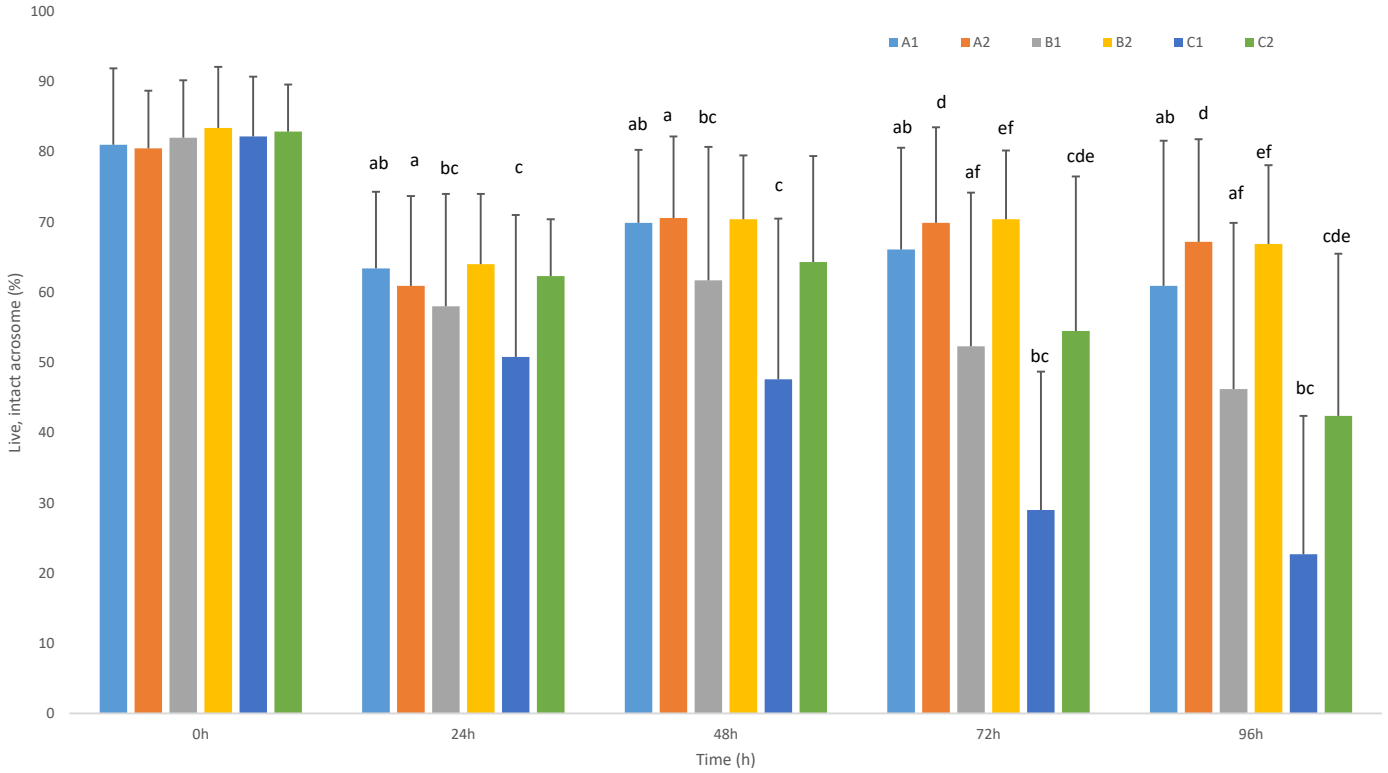

Fig. 2. Effect of sperm handling method single-layer centrifugation, sperm washing, or extension only) and extender (INRA82 or INRA96) on proportion of live acrosome intact spermatozoa during cooled storage for 96 hours (n = 24). Note: A1, single-layer centrifugation in INRA82; A2, single-layer centrifugation in INRA96; B1, sperm washing in INRA82; B2, sperm washing in INRA96; C1, extension in INRA82; C2 = extension in INRA96. Same letters indicate statistical significance: Note: A1, single-layer centrifugation in INRA82; A2, single-layer centrifugation in INRA96; B1, sperm washing in INRA82; B2, sperm washing in INRA96; C1, extension in INRA82; C2, extension in INRA96. Same letters indicate statistical significance: at 24 hours, a, b = $P < .01$, c = $P < .05$; at 48 hours a = $P < .01$, b, c $P < .001$; at 72hours a $P < .01$, b, c, d, e, f $P < .001$; at 96 hours a, b, c, d, e, f $P < .001$.

general, SLC and sperm washing in INRA96 gave samples of comparable acrosome status, although motility was better in the SLC samples at 24–72 hours than in the washed samples, and there was less DNA fragmentation in the SLC samples than in the washed samples. Interestingly, sperm washing in both types of extender showed a more deleterious effect on DNA fragmentation than controls and SLC samples.

Our results are in agreement with other studies comparing sperm washing and sperm extension, showing a beneficial effect of sperm washing on sperm motility [2]. Furthermore, the present results are in partial agreement with a previous study, in which SLC was found to result in better sperm motility than either sperm washing or extension, although no difference was observed between the latter two treatments [14].

The effect of SLC on sperm motility varies considerably between studies. In most cases, total and/or progressive motility is reported to be improved after SLC [20,21], or at least not adversely affected by SLC [22], due to exclusion of some immotile spermatozoa that have been unable to pass into the colloid. Differences between studies regarding whether or not there is an improvement may be due to differences in the centrifugation protocol, extender effects, chamber type, or the setting used for CASA instruments. In this study, there was a positive interaction between extender and SLC on maintenance of motility, as shown previously [20]. Of the extenders used in the latter trial, sperm motility was found to be retained best in INRA96. In the present study, there were no differences in total and progressive motility between SLC and sperm washing at various time points where the same extender was used.

Table 4
Proportion of abnormal spermatozoa (least squares means ± SEM; %) in different treatments (%) over time (n = 24).

Time	Treatment Group					
	A1	A2	B1	B2	C1	C2
0 h	30 ± 3	31 ± 3	36 ± 3	41 ± 3	35 ± 5	40 ± 5
24 h	31 ± 3	35 ± 3	37 ± 3	40 ± 3	37 ± 3	37 ± 3
48 h	44 ± 3	45 ± 3	43 ± 3	44 ± 3	42 ± 3	41 ± 3
72 h	43 ± 4	43 ± 5	48 ± 4	49 ± 5	43 ± 4	54 ± 4

Abbreviations: A1, SLC with INRA82; A2, SLC with INRA96; B1, sperm washing with INRA82; B2, sperm washing with INRA96; C1, extended in INRA 82; C2, extended in INRA96.
No significant differences among treatments. Significant interaction between time and treatment for 0 hours versus 48 hours and 24 hours versus 48 hours.

Table 5
Proportion of spermatozoa with abnormal heads in different treatments (unadjusted mean ± SD) over time.

Treatment	Time (h)				
	0	24	48	72	96
A1	29 ± 14	28 ± 15	41 ± 14	37 ± 14	29 ± 12
A2	27 ± 15	32 ± 14	42 ± 13	36 ± 15	30 ± 11
B1	29 ± 14	32 ± 16	37 ± 15	39 ± 12	27 ± 8
B2	35 ± 6	32 ± 14	40 ± 10	39 ± 15	33 ± 8
C1	32 ± 4	31 ± 7	35 ± 11	35 ± 6	29 ± 11
C2	26 ± 0	33 ± 10	36 ± 9	43 ± 22	24 ± 9

Abbreviations: A1, SLC with INRA82; A2, SLC with INRA96; B1, sperm washing with INRA82; B2, sperm washing with INRA96; C1, extended in INRA 82; C2, extended in INRA96.
No significant differences were detected among treatments.

Table 6
Proportion of spermatozoa with abnormal tails in different treatments (unadjusted mean ± SD) over time.

Treatment	Time (h)				
	0	24	48	72	96
A1	4 ± 2	4 ± 3	3 ± 2	5 ± 2	6 ± 2
A2	4 ± 2	2 ± 2	3 ± 3	2 ± 1	3 ± 2
B1	6 ± 2	5 ± 3	6 ± 3	7 ± 4	6 ± 3
B2	6 ± 3	5 ± 3	5 ± 4	7 ± 3	4 ± 2
C1	5 ± 4	6 ± 2	6 ± 2	6 ± 1	5 ± 3
C2	1 ± 9	4 ± 4	6 ± 3	7 ± 6	6 ± 3

Abbreviations: A1, SLC with INRA82; A2, SLC with INRA96; B1, sperm washing with INRA82; B2, sperm washing with INRA96; C1, extended in INRA 82; C2, extended in INRA96.
Statistical differences for A1 versus B1, $P < .01$; A2 versus B2, $P < .05$. Sperm samples washed in INRA82 had fewer tail defects than those washed in INRA96 ($P < .05$).

However, there were differences in ALH and BCF, with SLC-selected samples showing lower mean values of ALH and higher mean values of BCF than the washed samples. These results indicate that the spermatozoa had a more "progressive" pattern of motility after SLC, with smaller (lower ALH) but more frequent (higher BCF) head movements. Mean velocities were lower in the SLC and sperm washing treatments than in the extended group.

Sperm treatment can affect the appearance of abnormalities, particularly tail defects. It was surprising that there was a tendency for washing in INRA96 to be associated with more tail defects than in the other treatments. It has been shown previously that sperm samples selected by SLC have fewer morphological defects than controls, but this is the first time that the morphology of SLC and washed samples has been compared. Tail defects can be induced by using a hypo-osmolar extender [23], but this should not have been the case with INRA96, which has an osmolarity of approximately 320 mOsm according to the manufacturer.

The chromatin stability results presented here confirm previous studies in which chromatin integrity was monitored in SLC and extended samples [24], or in SLC and washed samples [19] for 48 hours. In all cases, the SLC groups had the lowest %DFI and retained these low levels of chromatin damage during the course of the experiment. By contrast, %DFI was seen to increase with storage in controls or in washed samples. Centrifugation without a colloid may result in the generation of reactive oxygen species that damage the spermatozoa [25]. However, the %DFI values were still relatively low after 96 hours in all treatment groups in the present study, which was surprising. Previous studies have reported higher %DFI in control samples than the values reported here [19].

In the present study, sperm membrane integrity was combined with evaluation of acrosome integrity, with the acrosome status of live spermatozoa being considered to be the most useful parameter for samples to be used for AI [26]. Sperm preparation method did not have an effect on acrosome status in live spermatozoa when evaluated immediately, but in SLC-selected samples, the proportion of live acrosome intact spermatozoa in INRA82 was higher than controls and sperm washing samples during storage. There were no differences between extenders for SLC-selected sperm samples. By contrast, extender had a significant effect in the sperm washing and control groups, where acrosome integrity in live spermatozoa was greater in INRA96 than in INRA82 for both treatments. Previous studies showed that membrane integrity in washed samples in INRA96 was lower than SLC-selected samples or controls [20], whereas Johannisson et al [24] reported that the proportion of membrane intact spermatozoa in Kenney's extender (equivalent to INRA82) was higher in SLC-selected samples than in controls. A

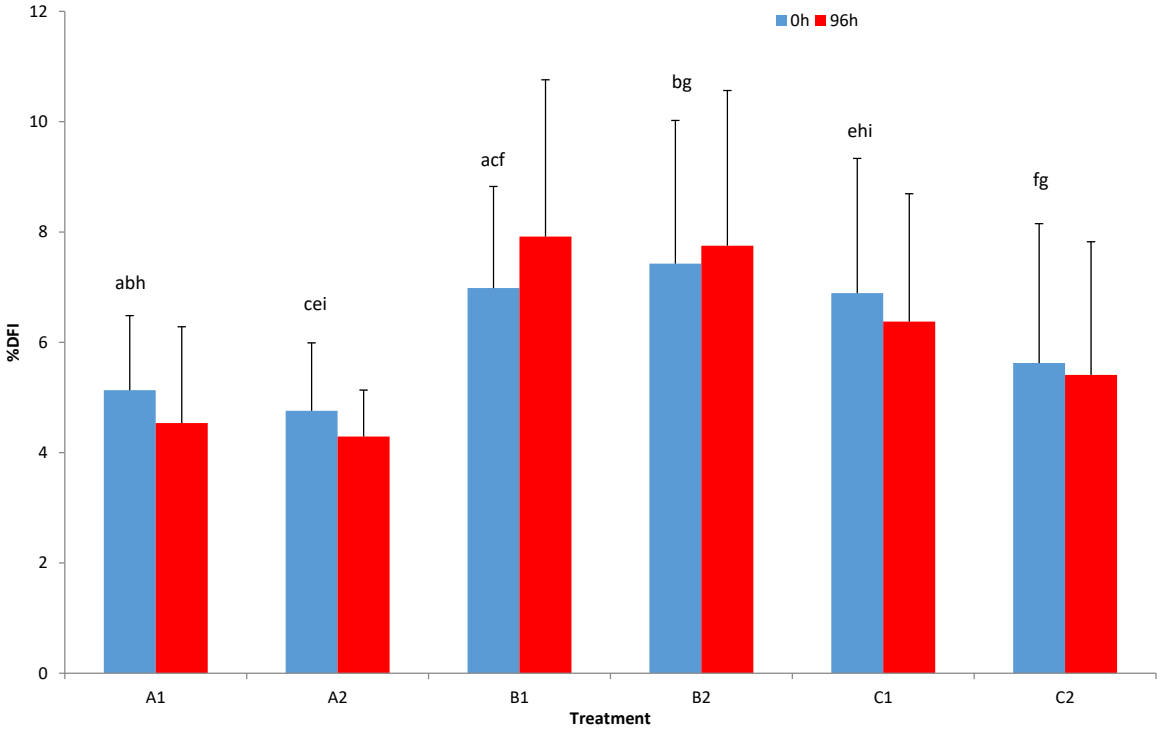

Fig. 3. Effect of sperm handling method (single-layer centrifugation, sperm washing, or extension only) and extender (INRA82 or INRA96) on mean (±SD) DNA Fragmentation index at 0 hours and 96 hours for single-layer centrifugation, sperm washing, and extension treatments (n = 24). A1, single-layer centrifugation in INRA82; A2, single-layer centrifugation in INRA96; B1, sperm washing in INRA82; B2, sperm washing in INRA96; C1, extension in INRA82; C2, extension in INRA96. Same letters indicate statistical significance as follows: Note: A1, single-layer centrifugation in INRA82; A2, single-layer centrifugation in INRA96; B1, sperm washing in INRA82; B2, sperm washing in INRA96; C1, extension in INRA82; C2, extension in INRA96. Similar letters indicate statistical significance as follows: a, b, c, g, i $P < .001$; f, g $P < .01$; h $P < .05$.

similar effect was observed by Al-Kass et al [27] for sperm samples in INRA96, stored cooled for 96 hours.

Few other studies have examined the effect of sperm preparation treatment on stallion sperm acrosome status. Early studies with density gradient centrifugation using Percoll for bull spermatozoa produced conflicting results on acrosome integrity, with some reports of an increased proportion of acrosome-reacted spermatozoa (e.g., [28]), whereas in others, there was an increase in the proportion of acrosome intact spermatozoa after colloid centrifugation [29]. These conflicting results may be due to the osmolarity of the colloid formulations. Early protocols used colloid formulations with an osmolarity similar to that of the oviducts, which may have contributed to an induction of the acrosome reaction. The Androcoll-E used in the present experiment has an osmolarity of 320 mOsm, which is higher than oviductal fluid [30] and therefore less likely to induce the acrosome reaction. Our results are comparable to those of Barrier-Battut et al [2], who concluded that the presence of SP in sperm samples in INRA96 (equivalent to our controls) increased the likelihood of spermatozoa undergoing the acrosome reaction when stimulated with calcium ionophore.

The interpretation of sperm acrosome status is somewhat problematic. It is established that only spermatozoa with intact acrosomes are potentially capable of fertilization, and therefore, theoretically, the higher the proportion of intact acrosomes in a sample initially, the better the chance of fertilization occurring [26]. However, only approximately one-third of stallion spermatozoa with intact acrosomes can undergo the acrosome reaction when stimulated with calcium ionophore [31], creating doubt about the usefulness of this assay as an indicator of stallion sperm fertility. It is possible that the induction protocol is not optimal, rather than that the acrosomes are not capable of reacting.

The fertility of semen extended in INRA82 and INRA96 for various times before insemination was compared previously [9]. It was concluded that pregnancy rates after AI were higher for semen extended in INRA96 than for semen extended in INRA82, although pregnancy rates after 72 hours storage were lower than after 24 hours.

In a previous AI trial using SLC-selected sperm samples from five stallions with fertility problems, sperm quality was improved, and pregnancies were obtained from all the stallions after preparation of ejaculates by SLC [32]. In a subsequent small AI trial with SLC-selected sperm samples from “normal” stallions, that is stallions without a fertility problem, in which sperm samples stored cooled for up to 96 hours were used for AI, high pregnancy rates were achieved after 48, 72 or 96 hours storage (69%) [13]. This result was very encouraging because most cooled stallion ejaculates have to be inseminated within 24–36 hours of semen collection to obtain a satisfactory pregnancy rate [1]. In a larger controlled trial, the pregnancy rate of SLC-selected sperm samples inseminated after 24 hours storage was significantly better than controls (69% vs. 45% for 160 inseminations) [33]. The latter AI trial included one of the stallions in the present study. For this stallion, the per cycle pregnancy rate for SLC and sperm washing samples in INRA96 were 70% and 50%, respectively, in keeping with the overall trend of results for the AI trial [33]. Thus, the *in vitro* sperm quality results reported in the present study support the fertility results reported in the AI trials, indicating that the better sperm quality observed in SLC-selected samples would be reflected in higher per cycle pregnancy rates.

5. Conclusions

The results of the present study indicate that SLC was an effective method to remove SP, often resulting in better sperm quality in the prepared samples than sperm washing. Sperm quality was better in either of these treatments than in the extended sample. Use of the chemically defined extender INRA96 maintained better sperm quality than INRA82, regardless of which sperm preparation was employed, with the exception of sperm tail morphological defects. These results suggest that SLC could be a useful adjunct to preparation of stallion semen for AI, removing SP without causing damage.

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