

Effect of various processing techniques on the quality of liquid-stored epididymal spermatozoa in stallion

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Abstract

Processing and storage of stallion epididymal spermatozoa are widely established procedures in equine practice worldwide. It represents the final opportunity to preserve the genetic potential of valuable breeding stallions that die unexpectedly or require castration for various reasons. Although some stallions retain a portion of progressively motile spermatozoa comparable to that of ejaculates collected using an artificial vagina, cryopreservation generally results in reduced progressive motility and, consequently, reduced fertilizing potential of the insemination dose. The aim of this study was to evaluate the effect of a 24-h interval between castration and epididymal sperm recovery, and to assess the efficiency of different centrifugation procedures and storage in two extenders (INRA 96, EquiPlus) used for cooled stallion insemination doses. Sperm motility parameters were analysed using a CASA system, and the resulting data were statistically evaluated using a linear mixed-effects model. The results showed that samples processed with INRA 96 had significantly higher values for most evaluated CASA parameters than those processed with EquiPlus. While the effects of storage conditions and centrifugation were not consistent across all evaluated parameters, extender type had the most pronounced effect on sperm motility and kinematic characteristics.

Equine, retrograde epididymal flush, CASA, cushion fluid, centrifugation

In equine assisted reproduction, the recovery of epididymal spermatozoa is considered one of the last available options for preserving the genetic potential of valuable stallions affected by obstruction of the excurrent genital ducts or those that have undergone castration, required emergency euthanasia, or died suddenly as a result of acute or chronic disease (Mislei et al. 2016; Miró et al. 2020; Podico and Canisso 2022; Ram et al. 2024).

Retrograde epididymal flushing is regarded as the most effective (Guimarães et al. 2012) and, at the same time, the method most routinely used in practice for the recovery of epididymal spermatozoa. Following castration, an intravenous catheter is inserted into the ductus deferens, and the contents of the cauda epididymidis are expelled using a suitable extender and subsequently processed (Podico and Canisso 2022). Mislei et al. (2016) reported that a high proportion of spermatozoa contained within the cauda epididymidis may exhibit progressive motility (Morris et al. 2002) and fertility (Monteiro et al. 2011) comparable to those of spermatozoa obtained from stallion ejaculates collected using an artificial vagina.

The preservation of functional characteristics of epididymal spermatozoa after flushing and processing may, however, be influenced by several factors. Guimarães et al. (2012) and Ram et al. (2024) emphasized that adequate conditions for the handling and processing of epididymal tissue cannot always be ensured at the site of stallion castration. Therefore, a box with a cooling pack is commonly used for transport of the castrated testis-epididymis complex, allowing preservation of the biological material at approximately 4 °C for 24–48 h

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(Bruemmer 2006; Mislei et al. 2016; Ram et al. 2024). Some studies have reported maintenance of sperm quality for up to 96 h under these conditions (James et al. 2002; Miró et al. 2020). Monteiro et al. (2011) and Guimarães et al. (2012) showed that the functional capacity of epididymal spermatozoa prior to cryopreservation is not affected by whether the cooled testis-epididymis complex or spermatozoa flushed immediately after castration are transported to the laboratory. However, given the limited number of available studies, this area still requires further investigation.

Current attention has also focused on the effects of centrifugation and sperm processing procedures on sperm motility and potential fertility (Ellerbrock et al. 2017), as well as on the composition of the extender and the presence of antimicrobial agents (Miró et al. 2020; Lehmann et al. 2021; Ram et al. 2024; Gil et al. 2026).

Published studies to date, including those conducted at our institution, have focused primarily on the development and optimization of epididymal sperm cryopreservation (Mráčková et al. 2015; Mráčková et al. 2021; Andrllová et al. 2023). However, storage of epididymal spermatozoa under cooled conditions at approximately 4 °C also represents an important area of research, both before the freezing process and in the preparation of chilled insemination doses.

The aim of this study was to compare computer assisted sperm analysis (CASA)-derived motility parameters of epididymal spermatozoa flushed from the cauda epididymidis immediately after castration and stored for 24 h at 4 °C with those of spermatozoa stored for 24 h within the chilled testis-epididymis complex. A further objective was to compare the effects of different centrifugation methods applied to these samples in two commercial extenders (INRA 96 and EquiPlus) on the quality of stallion epididymal spermatozoa.

Materials and Methods

Animals and sample collection

Epididymal sperm samples included in this study were obtained from epididymides (n = 20) collected during castration of clinically healthy stallions (n = 10) of various breeds. The stallions were castrated between 2 and 7 years of age as part of routine clinical practice at the Equine Clinic, VETUNI Brno, Czech Republic. All stallions had both testes fully descended into the scrotum and had not previously been used for breeding. Immediately after castration performed under general injectable anaesthesia (xylazine, ketamine, diazepam, guaifenesin) without local intratesticular anaesthesia, both excised testes with epididymides and ligated ductus deferens were transported to a nearby laboratory for further evaluation and processing.

Sample processing

Before processing, the testes with epididymides were rinsed with sterile physiological saline pre-warmed to 37 °C and macroscopically examined to exclude morphological abnormalities and size discrepancies. One epididymis was randomly selected and processed at room temperature, and the recovered epididymal sperm samples were evaluated within 60 min of castration (the length of castration of both testicles, transport to laboratory, testicles and epididymis processing), whereas the second was cleaned, stored in a refrigerator intact testis-epididymis complex at 4 °C, and flushed after 24 h.

Epididymal spermatozoa were recovered from the ductus deferens and cauda epididymidis by routine retrograde flushing (Andrllová et al. 2023) using 20 ml of pre-warmed (37 °C) flushing medium (Ringer Solution Ecoflac Plus, B. Braun Medical, Melsungen, Germany) with flushing performed immediately after castration. When flushing was done the next day, the temperature of the medium was the same as the epididymidis (4 °C). Each flush underwent routine macroscopic evaluation and sperm morphology assessment, followed by microscopic estimation of total and progressive motility (%). A minimum total motility of 10% was required for inclusion in the study.

Each recovered sperm sample was divided into 10 tubes containing 2 ml each, which were then processed in pairs using five different methods (C–C4). These methods were devised specifically for this present experiment design, drawing on published protocols for stallion sperm processing (Waite et al. 2008; dos Santos et al. 2017; Ellerbrock et al. 2017; Zabala et al. 2024; Gil et al. 2026) and routine practice in our laboratory. The resulting split-sample design allowed simultaneous assessment of the effects of the experimental factors on sperm motility parameters while minimizing the influence of variability among stallion and epididymides. A centrifugation extender (Centrifuge medium for equine semen, Minitube International, Tiefenbach, Germany) and cushion fluid (CushionFluid, Minitube International) were used in the study. The latter is a high-

molecular-weight, iodoxanol-based colloid that forms a dense layer beneath the sperm sample at the bottom of the centrifuge tube, thereby limiting sperm compaction and associated mechanical damage and improving sperm recovery (Waite et al. 2008; dos Santos et al. 2017).

Methods of epididymal flush processing

- C – non-centrifuged control sample
- C1 – centrifugation of the flush without further dilution
- C2 – centrifugation of the flush diluted 1:1 with centrifugation extender
- C3 – centrifugation of the flush diluted 1:1 with centrifugation extender and with 1 ml of cushion fluid at the bottom of the tube
- C4 – centrifugation of undiluted flush with 1 ml of cushion fluid at the bottom of the tube

After centrifugation ($1,000 \times g$ for 20 min), the supernatant and cushion fluid were removed from all centrifuged samples. The sperm pellet was then slowly resuspended in two different commercial extenders intended for cooled stallion insemination doses. Within each of the five processing methods, one tube was diluted in INRA 96 extender (IVM Technologies, Marseille, France) and the other in EquiPlus (Minitube International) to a final concentration of approximately 50×10^6 spermatozoa/ml (range $45\text{--}55 \times 10^6$ spermatozoa/ml), which allowed evaluation of the samples using the CASA system.

Sample analysis

Sperm motility was evaluated in all samples using a computer-assisted sperm analysis system CASA (HTM-CEROS, version 12.3; Hamilton Thorne, Beverly, MA, USA) coupled to an Eclipse E200 microscope (Nikon Instruments, Tokyo, Japan). Diluted samples were loaded into 20- μm Leja chambers (Leja Products B.V., Hoofddorp, Netherlands). Total and progressive motility, together with selected kinematic characteristics, were assessed at 60 Hz using 40 frames per field. Progressive motility was defined as $\text{VAP} \geq 50 \mu\text{m/s}$ and $\text{STR} \geq 75\%$, whereas slow spermatozoa were classified using cut-off values of $\text{VAP} \leq 20 \mu\text{m/s}$ and $\text{VSL} \leq 0 \mu\text{m/s}$. A minimum cell size of 2 pixels, minimum contrast of 45, and default cell size of 3 pixels were applied. The CASA settings were kept constant throughout the study.

Samples recovered from the first epididymis (A) processed and analysed immediately after castration on Day 1 (Immediate) were subsequently stored at 4°C for repeated analysis after 24 h (Liquid_24 h). The second measurement was performed in the same manner as the first, with the only difference being that refrigerated samples were analysed after 5 min of pre-warming (2.5 ml sample volume) in a heating block MaXtable H10 (DaiHan Scientific, 2015, Seoul, South Korea) at 37°C (Lehmann et al. 2022). In the second testis with epididymis (B), the same epididymal sperm recovery technique was applied after 24 h storage of the whole testis-epididymis complex at 4°C (Epididymis_24h). Processing, evaluation, and analysis were performed in the same manner, including the pre-warming step, in order to ensure comparability among samples.

Statistical analysis

Statistical analysis of the obtained data was performed in R software (R Foundation for Statistical Computing, Vienna, Austria) using the R Commander graphical interface. The sperm quality variables included in the analysis were total motility (TM), progressive motility (PM), straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), and straightness (STR). The effects of centrifugation method, extender type, and storage condition were evaluated using linear mixed-effects models (LMMs).

The storage conditions variable included three experimental situations: immediate sperm recovery (Immediate), immediate recovery followed by 24 h storage of the sperm flush at 4°C (Liquid_24h), and sperm recovery performed only after 24 h storage of the whole testis with epididymis at 4°C (Epididymis_24h). Centrifugation method, extender type, and storage condition were included in the model as fixed effects. In each model, the Intercept represented the estimated value when all fixed effects were at their reference levels. Stallion identity and testis nested within stallion were considered random effects to account for repeated measurements and biological variability among individual animals.

Comparisons among centrifugation methods and storage conditions were performed using pairwise comparisons with Tukey's correction, and statistical significance was set at $P < 0.05$.

Results

For greater clarity and easier interpretation of the relationships among the applied methods of epididymal sperm processing, the commercial extenders used, and the storage conditions, the results are presented according to the individual motility and sperm motion parameters evaluated. Differences among individual stallion were not examined, as stallion was included as a random effect.

Total motility

Total motility was not significantly affected by the centrifugation method. In the LMM, none of the centrifugation techniques (C1–C4) differed significantly from the untreated control group C (all $P > 0.05$). Likewise, subsequent pairwise comparisons among the individual centrifugation methods revealed no significant differences. In contrast, the extender type had a significant effect, with samples diluted in INRA 96 showing higher TM values than samples processed in the reference extender EquiPlus ($P < 0.001$). Storage condition also had a significant effect, as the Liquid_24h group showed significantly lower total motility than the Immediate group ($P < 0.001$). Tukey's *post hoc* analysis confirmed a significant difference only between the Immediate and Liquid_24h groups ($P < 0.001$), whereas no significant differences were found between Immediate and Epididymis_24 or between Liquid_24h and Epididymis_24. Detailed results are presented in Tables 1, 2, 4, and in Figs 1A, 3A.

Progressive motility

Progressive motility was not significantly affected by centrifugation method, and in the LMM analysis none of the centrifugation groups (C1–C4) differed significantly from the control group. Although group C3 in the main model indicated higher PM compared with the control, subsequent pairwise comparisons did not confirm any statistically significant differences (all $P > 0.05$). As in TM, extender type had a significant effect on PM. Dilution of epididymal spermatozoa in INRA 96 resulted in significantly higher progressive motility than dilution in the reference extender EquiPlus ($P < 0.001$). No significant differences were found among the Immediate, Liquid_24h, and Epididymis_24 groups, either in the main model or in the *post hoc* analysis ($P > 0.05$). Detailed data are presented in Tables 1, 2, 4 and in Figs 1B, 3B.

Table 1. Effects of centrifugation method, extender type and storage condition on total and progressive motility of epididymal spermatozoa.

Variable	Effect	Estimate	SE	df	t value	P value
Progressive motility	Intercept	12.987	3.886	24.498	3.342	0.00267
	C1 vs C	2.483	1.994	274.19	1.245	0.21403
	C2 vs C	2.917	1.994	274.19	1.463	0.14467
	C3 vs C	4.533	1.994	274.19	2.274	0.02377
	C4 vs C	2.417	1.994	274.19	1.212	0.22655
	INRA vs EquiPlus	13.147	1.261	274.19	10.425	< 0.0001
	Liquid_24h vs Immediate	-2.560	1.544	274.19	-1.658	0.09856
Total motility	Epididymis_24 vs Immediate	-3.970	4.672	9.521	-0.85	0.41629
	Intercept	54.973	7.799	17.521	7.048	< 0.0001
	C1 vs C	-0.533	2.903	273.965	-0.184	0.854
	C2 vs C	-0.633	2.903	273.965	-0.218	0.827
	C3 vs C	2.217	2.903	273.965	0.763	0.446
	C4 vs C	-1.283	2.903	273.965	-0.442	0.659
	INRA vs EquiPlus	16.867	1.836	273.965	9.186	< 0.0001
	Liquid_24h vs Immediate	-12.460	2.249	273.965	-5.54	< 0.0001
	Epididymis_24 vs Immediate	-6.660	7.859	9.383	-0.847	0.418

C = non-centrifuged control; C1–C4 = centrifugation methods described in Materials and Methods; SE = standard error; df = degrees of freedom. C, EquiPlus, and Immediate were the reference levels for centrifugation method, extender, and storage condition respectively. For each outcome, the Intercept represents the estimated motility (%) for this reference combination C, EquiPlus, and Immediate. All non-intercept estimates represent model-estimated differences from the corresponding reference level in percentage points whereas all other estimates represent differences in percentage points; positive estimates indicate higher motility and negative estimates indicate lower motility relative to the reference level. SE values are expressed on the corresponding percentage-point scale, whereas t statistics and P values are unitless. The models included stallion and epididymis nested within stallion as random effects. P values represent unadjusted model coefficient tests; Tukey-adjusted comparisons for factors with more than two levels are reported in Tables 2–4 from the corresponding reference level.

Straight-line velocity

Straight-line velocity was not significantly affected by centrifugation method, as neither the LMM analysis nor Tukey's *post hoc* pairwise comparisons showed significant differences among the centrifugation groups (all $P > 0.05$). In contrast, extender type had a significant effect on VSL, with samples diluted in INRA 96 showing higher values than samples processed in EquiPlus ($P < 0.001$). Storage condition also significantly

Table 2. Estimated marginal means and Tukey-adjusted pairwise comparisons for total and progressive motility according to storage condition.

Estimated marginal means					
Variable	Storage condition	EMM	SE	df	95% CI
Progressive motility	Immediate	22.00	3.62	18.34	14.4–29.6
	Liquid_24h	19.50	3.62	18.34	11.9–27.1
	Epididymis_24	18.10	3.62	18.34	10.5–25.7
Total motility	Immediate	63.40	7.52	15.20	47.3–79.4
	Liquid_24h	50.90	7.52	15.20	34.9–66.9
	Epididymis_24	56.70	7.52	15.20	40.7–72.7
Tukey-adjusted pairwise comparisons					
Variable	Comparison	Difference	SE	df	<i>P</i> value
Progressive motility	Immediate vs Liquid_24h	2.56	1.54	274.01	0.2236
	Immediate vs Epididymis_24	3.97	4.67	9.52	0.6827
	Liquid_24h vs Epididymis_24	1.41	4.67	9.52	0.9513
Total motility	Immediate vs Liquid_24h	12.46	2.25	274	<0.0001***
	Immediate vs Epididymis_24	6.66	7.86	9.38	0.6842
	Liquid_24h vs Epididymis_24	–5.80	7.86	9.38	0.7477

EMM = estimated marginal mean; CI = confidence interval; SE = standard error; df = degrees of freedom. EMMs are expressed as percentages, and pairwise differences are expressed in percentage points and were calculated as the first condition minus the second. *P* values for pairwise comparisons were adjusted using Tukey's method. Significant differences are indicated by asterisks (* $P < 0.05$, *** $P < 0.001$).

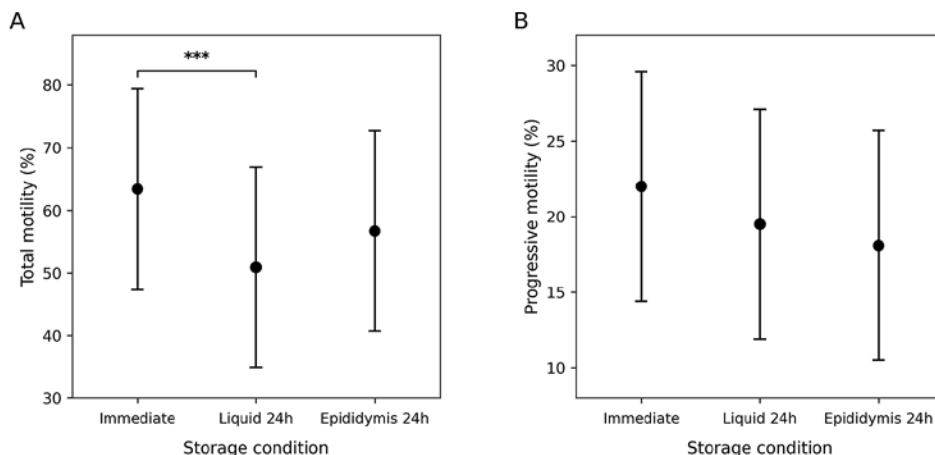


Fig. 1. Effect of storage condition on total and progressive motility of epididymal spermatozoa. Estimated marginal means of (A) total motility (TM, %) and (B) progressive motility (PM, %) in samples processed immediately after epididymal recovery (Immediate), after 24 h storage of diluted spermatozoa in liquid form (Liquid_24h), or after 24 h storage of the cauda epididymidis before sperm recovery (Epididymis_24). Error bars represent 95% confidence intervals. The bracket and asterisks in panel A denote a significant Tukey-adjusted pairwise difference between Immediate and Liquid_24 (***) ($P < 0.001$).

influenced VSL. The Immediate group showed significantly higher VSL than the Liquid_24h group ($P < 0.001$), whereas no significant differences were found between Immediate and Epididymis_24 or between Liquid_24h and Epididymis_24. Detailed results are presented in Tables 3, 4 and Figs 2A, 3C.

Average path velocity

In the case of VAP, a significantly lower value was detected in group C2 compared to the control group ($P < 0.05$), but pairwise comparisons among the individual centrifugation methods did not confirm any significant differences. Extender type did not significantly affect VAP ($P > 0.05$). *Post hoc* pairwise comparisons showed that VAP was significantly higher in the Immediate group than in the Liquid_24h group ($P < 0.001$) and also significantly higher in the Epididymis_24 group than in the Liquid_24h group ($P < 0.05$). Detailed results are presented in Tables 3 and 4 and Fig. 2B.

Table 3. Effects of centrifugation method, extender type and storage condition on sperm motion parameters of epididymal spermatozoa.

Estimated marginal means					
Variable	Storage condition	EMM	SE	df	95% CI
VSL	Immediate	39.70	2.26	16.82	34.9–44.4
	Liquid_24h	33.20	2.26	16.82	28.4–38.0
	Epididymis_24	37.00	2.26	16.82	32.2–41.8
VAP	Immediate	57.70	3.12	14.27	51.0–64.4
	Liquid_24h	45.70	3.12	14.27	39.1–52.4
	Epididymis_24	55.30	3.12	14.27	48.6–62.0
VCL	Immediate	120.10	5.86	14.38	107.6–133.0
	Liquid_24h	97.10	5.86	14.38	84.6–110.0
	Epididymis_24	116.90	5.86	14.38	104.3–129.0
STR	Immediate	67.60	1.87	19.07	63.7–71.5
	Liquid_24h	70.00	1.87	19.07	66.1–73.9
	Epididymis_24	65.10	1.87	19.07	61.2–69.0
Tukey-adjusted pairwise comparisons					
Variable	Comparison	Difference	SE	df	<i>P</i> value
VSL	Immediate vs Liquid_24h	6.49	1.00	274.01	< 0.0001***
	Immediate vs Epididymis_24	2.68	2.59	9.73	0.5717
	Liquid_24h vs Epididymis_24	–3.81	2.59	9.73	0.3445
VAP	Immediate vs Liquid_24h	11.99	1.13	274.00	< 0.0001***
	Immediate vs Epididymis_24	2.42	2.98	9.70	0.7047
	Liquid_24h vs Epididymis_24	–9.57	2.98	9.70	0.0239*
VCL	Immediate vs Liquid_24h	23.00	2.05	274.01	< 0.0001***
	Immediate vs Epididymis_24	3.24	5.67	9.62	0.8379
	Liquid_24h vs Epididymis_24	–19.76	5.67	9.62	0.0156*
STR	Immediate vs Liquid_24h	–2.37	1.00	274.03	0.0488*
	Immediate vs Epididymis_24	2.48	2.45	9.83	0.5877
	Liquid_24h vs Epididymis_24	4.85	2.45	9.83	0.1695

SE = standard error; df = degrees of freedom. VSL, VAP, and VCL EMMs and pairwise differences are expressed in $\mu\text{m/s}$. STR EMMs are expressed as percentages, whereas STR pairwise differences are expressed in percentage points. Pairwise differences were calculated as the first condition minus the second, and *P* values were adjusted using Tukey's method. Significant differences are indicated by asterisks (* $P < 0.05$, *** $P < 0.001$).

Curvilinear velocity

Curvilinear velocity was significantly affected by centrifugation method, with group C2 showing significantly lower VCL than the control group ($P < 0.05$); this difference was also confirmed by Tukey's *post hoc* pairwise comparison. The remaining centrifugation groups did not differ significantly from the control. Curvilinear velocity was also significantly affected by extender type and was higher in samples processed in INRA 96 than in samples processed in EquiPlus ($P < 0.001$). Storage condition also had a significant effect on VCL. *Post hoc* pairwise comparisons showed that VCL was significantly higher in the Immediate group than in the Liquid_24h group ($P < 0.001$) and also significantly higher in the Epididymis_24 group than in the Liquid_24h group ($P < 0.05$), whereas no significant difference was found between Immediate and Epididymis_24. Results are presented in Tables 3, 4 and Figs 2C, 3D.

Table 4. Estimated marginal means of sperm motility and kinematic parameters by centrifugation method, with Tukey-adjusted comparisons with the non-centrifuged control.

Variable	Estimated marginal means				
	C	C1	C2	C3	C4
Progressive motility	17.4	19.9	20.3	21.9	19.8
Total motility	57.0	56.5	56.4	59.2	55.8
VSL	36.1	37.2	35.4	37.2	37.1
VAP	54.0	52.9	50.5	53	54.2
VCL	114	111	106	111	113
STR	65.2	68.8	68.5	68.7	66.6
Tukey-adjusted comparisons with the non-centrifuged group					
Variable	Comparison		Difference	P value	
Progressive motility	C1 vs C		2.4833	0.7247	
	C2 vs C		2.9167	0.5876	
	C3 vs C		4.5333	0.1567	
	C4 vs C		2.4167	0.7444	
Total motility	C1 vs C		-0.5330	0.9997	
	C2 vs C		-0.6330	0.9995	
	C3 vs C		2.2170	0.9408	
	C4 vs C		-1.2830	0.9921	
VSL	C1 vs C		1.0567	0.9251	
	C2 vs C		-0.7433	0.9786	
	C3 vs C		1.0800	0.9194	
	C4 vs C		0.9783	0.9426	
VAP	C1 vs C		-1.0383	0.9542	
	C2 vs C		-3.4483	0.1305	
	C3 vs C		-0.9917	0.9611	
	C4 vs C		0.1917	0.9999	
VCL	C1 vs C		-3.0520	0.7782	
	C2 vs C		-8.0400	0.0219*	
	C3 vs C		-3.3530	0.7120	
	C4 vs C		-1.1530	0.9925	
STR	C1 vs C		3.5167	0.0537	
	C2 vs C		3.2667	0.0878	
	C3 vs C		3.4833	0.0575	
	C4 vs C		1.8333	0.8219	

C = non-centrifuged control; C1–C4 = centrifugation methods described in Materials and Methods; EMM = estimated marginal mean; VSL = straight-line velocity; VAP = average path velocity; VCL = curvilinear velocity; STR = straightness. Progressive motility, total motility, and STR EMMs are expressed as percentages, whereas their differences are expressed in percentage points. VSL, VAP, and VCL EMMs and differences are expressed in $\mu\text{m/s}$. Differences were calculated as the respective centrifugation method minus C. Only Tukey-adjusted comparisons with C are shown. Significant differences are indicated by asterisks (* $P < 0.05$, *** $P < 0.001$).

Straightness

In the LMM analysis, centrifugation groups C1, C2, and C3 showed higher STR values than the control group, but Tukey's pairwise comparisons among the individual methods did not confirm these differences as statistically significant. Group C4 did not differ significantly from the control. Straightness was also significantly affected by extender type. Samples diluted in INRA 96 showed higher STR values than samples processed in EquiPlus ($P < 0.001$). Storage condition also had a significant effect on STR. *Post hoc* pairwise comparisons showed that STR was significantly higher in the Liquid_24h group than in the Immediate group ($P < 0.05$), whereas no significant differences were found between Immediate and Epididymis_24 or between Liquid_24h and Epididymis_24. Results are presented in Tables 3, 4 and Figs 2D, 3E.

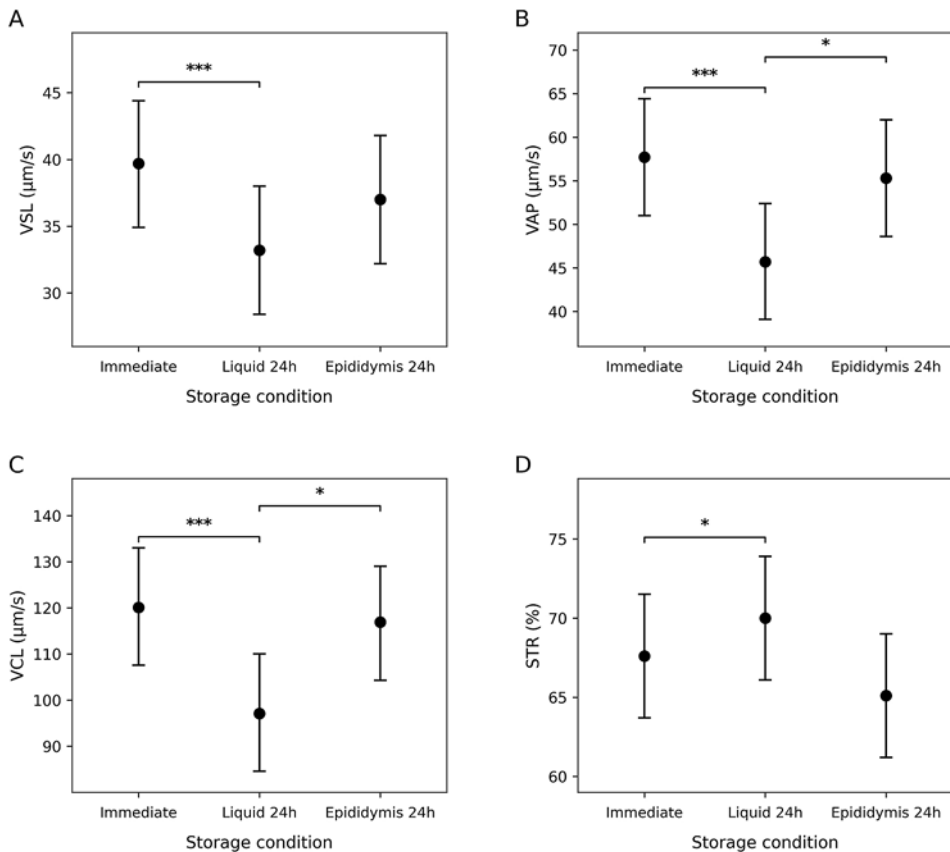


Fig. 2. Effect of storage condition on sperm motion parameters of epididymal spermatozoa. Estimated marginal means of (A) straight-line velocity (VSL, μm), (B) average path velocity (VAP, μm), (C) curvilinear velocity (VCL, μm), and (D) straightness (STR, %) in samples processed immediately after epididymal recovery (Immediate), after 24 h storage of diluted spermatozoa in liquid form (Liquid_24h), or after 24 h storage of the cauda epididymidis before sperm recovery (Epididymis_24). Error bars represent 95% confidence intervals. The bracket and asterisks in panel denote a statistically significant Tukey-adjusted pairwise difference (* $P < 0.05$, *** $P < 0.001$).

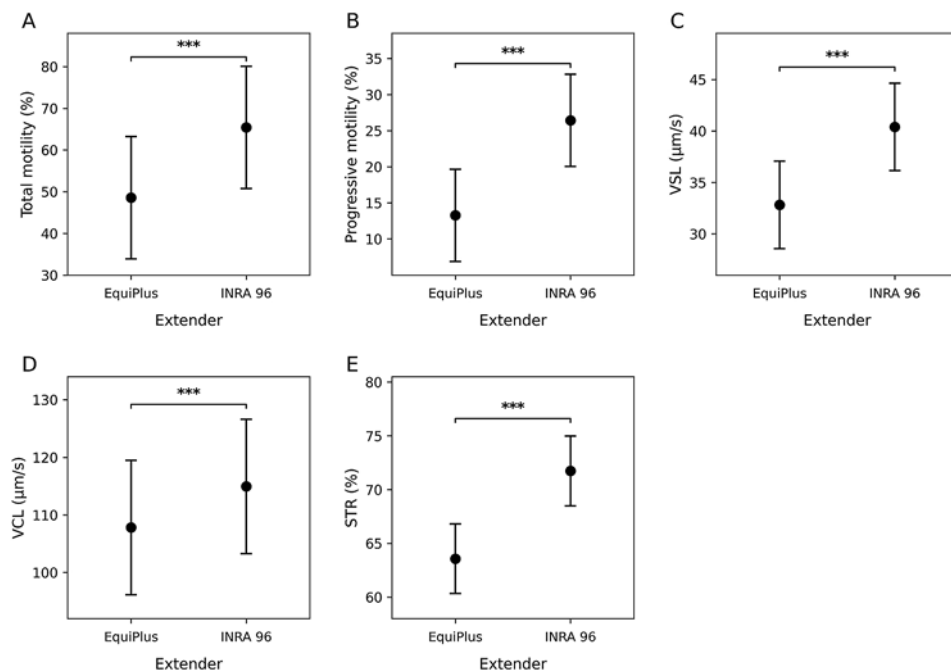


Fig. 3. Effect of extender type on motility and sperm motion parameters of epididymal spermatozoa. Estimated marginal means of (A) total motility (TM, %), (B) progressive motility (TM, %), (C) straight-line velocity (VSL, μm), (D) curvilinear velocity (VCL, μm), and (E) straightness (STR, %) in samples processed using EquiPlus or INRA 96. Error bars represent 95% confidence intervals. The bracket and asterisks in panel A denote a significant Tukey-adjusted pairwise difference between extenders (** $P < 0.001$).

Discussion

Our study evaluated several factors that may affect the quality and motility indicators of epididymal spermatozoa, specifically TM, PM, VSL, VAP, VCL, and STR. The main aim was to assess whether the storage method, the centrifugation procedures used, and commercial semen extenders influence the motility indicators of epididymal spermatozoa. Given the reported association of PM with fertility and viability of fresh and frozen stallion semen (Egyptien et al. 2023) and the routine use of TM in semen evaluation (Brinsko et al. 2011; Waberski et al. 2022), PM and TM were considered the primary motility outcomes, whereas VCL, VSL, VAP, and STR were considered supplementary CASA parameters. Statistically significant differences in these supplementary parameters should therefore be interpreted as changes in sperm kinematics rather than direct evidence of a corresponding effect on fertility.

The first part of the experiment focused on the effect of storage conditions. However, the results of our study on epididymal spermatozoa do not provide a fully consistent conclusion, as no significant differences were found in most variables (TM, PM, VSL, STR) between samples recovered by retrograde flushing immediately after castration and stored in liquid form at 4 °C and samples recovered only after 24 h of storage of the testis-epididymis complex. This finding is consistent with the conclusions of Monteiro et al. (2011) and Guimarães et al. (2012), while Monteiro et al. (2011) also reported preservation of fertilizing potential in both methods of epididymal sperm storage prior to cryopreservation. Our results therefore support the assumption that transport of the

chilled testis-epididymis complex for 24 h from the site of castration does not necessarily negatively affect epididymal sperm motility and kinematics. Some authors have emphasized that, in cases where retrograde flushing can be performed under field conditions, transport of the flush alone may represent a more advantageous alternative from both logistical and economic perspectives (Guimarães et al. 2012). However, inappropriate manipulation and separation of the ductus deferens may result in blood contamination of the flush, thereby compromising sperm quality. Therefore, for routine practice, retrograde flushing performed in the laboratory by experienced personnel may be preferable.

The second part of the study focused on the effect of centrifugation. While centrifugation associated with the removal of seminal plasma is a routine part of ejaculate processing (Kelley 2024; Medica et al. 2024), its use in epididymal spermatozoa is more specific and is generally applied mainly when retrograde flushing is performed using extenders intended for the preparation of cooled insemination doses (Podico and Canisso 2022). In our study, only a single variable (VCL) was significantly affected by the selected centrifugation method (C2), but this finding cannot be considered conclusive when interpreted in isolation, without a concomitant increase in PM. The stability of the remaining motility parameters suggests that, under the conditions used, the selected centrifugation protocol does not represent a major factor affecting the motility and quality of epididymal spermatozoa, which is consistent with the conclusions of Ellerbrock et al. (2017).

The main finding of our study was that extender type significantly affected almost all evaluated motility parameters of epididymal spermatozoa. Values were generally higher in samples processed with INRA 96 than with EquiPlus, suggesting that extender composition plays an important role in maintaining the motility characteristics of both ejaculated and epididymal spermatozoa (Miró et al. 2020; Gil et al. 2026). Comparable findings in cooled ejaculated stallion semen were reported by Vokrouhliková et al. (2021). However, the precise mechanism underlying these differences cannot be determined from the present study, partly because the complete quantitative compositions of the two commercial products are not publicly disclosed. The higher motility values may therefore reflect differences in the native phosphocaseinate fraction, energy substrates, buffering systems, or antimicrobial supplementation. For example, Batellier et al. (2000) reported a direct protective effect of native phosphocaseinate on stallion spermatozoa.

Given the large number of commercially available extenders, future studies should also compare INRA 96 with additional formulations, including media with modified or enriched composition (Mislei et al. 2016; Neuhauser et al. 2018; Nunes et al. 2025; Gil et al. 2026), which may further influence sperm motility.

To compare the two extenders, a neutral flushing solution was used before centrifugation and subsequent dilution in the respective extenders. Ringer B. Braun appears to be a suitable option for short-term preservation of epididymal sperm quality (Lehmann et al. 2022).

Several limitations should be considered when interpreting our results. Sperm quality variables were assessed solely on the basis of instrument-measured motility indicators, which may reflect the fertilizing potential to some extent, but do not by themselves allow a definitive conclusion regarding actual fertility (Love 2011). To confirm the biological relevance of the observed differences, future studies should therefore complement CASA-derived parameters with data on pregnancy rates in mares inseminated with cooled doses, or alternatively with evaluation of sperm use in ICSI. In addition, the relatively small number of stallions may have reduced the statistical power to detect smaller effects and limited the assessment of between stallion variability. The findings should therefore be confirmed in a larger population including stallions of different breeds and a broader age range.

This study provides new, although still limited, information on the use of commercial extenders for the processing and short-term chilled storage of epididymal spermatozoa.

While the effects of storage conditions and centrifugation were not uniform across all evaluated parameters, the extender type significantly influenced most motility characteristics, with higher values observed in samples processed with INRA 96 than in those processed with EquiPlus. Further fertility-oriented studies will be required to confirm the practical relevance of these findings.

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