Final Degree Project in Veterinary

Title: Fresh and post-thaw sperm quality after epididymal retrograde flushing in dogs and rabbits

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Abstract

Cryopreservation of spermatozoa is an essential tool in reproductive biotechnology, but the freezing and thawing process can significantly impact sperm quality, particularly in epididymal spermatozoa, which are more sensitive to cryodamage. This study aimed to evaluate the effects of two different TRIS-based extenders and two thawing protocols on the post-thaw quality of epididymal spermatozoa collected from dogs and rabbits. Epididymal sperm was collected post-castration using retrograde flushing. Fresh sperm quality was assessed by evaluating sperm motility, vigor, viability, acrosome integrity and sperm abnormalities. The samples were then cryopreserved using two extenders with different TRIS concentrations (198 mM and 248 mM). Frozen samples were thawed using two different protocols: 37°C for 30 seconds and 70°C for 8 seconds. In dogs, spermatozoa preserved relatively high post-thaw quality, particularly in the group using the higher TRIS concentration and thawed at 70 °C. Rabbit spermatozoa demonstrated a marked decrease in all quality parameters after thawing, regardless of protocol. However, 37 °C thawing condition yielded slightly better motility outcomes. This study provides valuable insights into the impact of extender composition and thawing protocols on epididymal sperm preservation in dogs and rabbits. Understanding these factors can contribute to optimizing cryopreservation techniques for improved sperm functionality in both species.

Keywords: Dog, Rabbit, Epididymal sperm, Cryopreservation, Sperm quality.

1. INTRODUCTION

Modern reproductive technologies play a crucial role in the preservation of genetic material in domestic and wild animals. One significant approach is the use of spermatozoa retrieved from the cauda epididymis after castration or the sudden death of males. This method is particularly relevant for conserving rare species, increasing genetic diversity in populations, and extending the reproductive potential of valuable breeding animals (Hori *et al.,* 2011; Nishijima *et al.,* 2021). The use of epididymal sperm is essential in cases where traditional semen collection methods are not feasible. However, freezing and storing epididymal sperm presents several challenges, including reduced cryotolerance, as well as susceptibility to oxidative and osmotic stress, which lead to damage to membranes and acrosomes (Galarza *et al.,* 2021; Pulkowska-Bluj & Trzcińska, 2025). Despite these limitations, the successful application of frozen epididymal sperm in artificial insemination confirms the potential of this method (Hori *et al.,* 2004; Klinc *et al.,* 2005).

Dogs and rabbits were selected for this study due to their accessibility through routine veterinary castration procedures and their established value as experimental models in both reproductive and biomedical research. Rabbits are widely utilized in agriculture and laboratory settings, particularly for antibody production and the maintenance of transgenic lines. Cryopreservation of rabbit sperm facilitates long-term genetic preservation while reducing the need for continuous breeding programs. However, rabbit sperm is known to be highly sensitive to cryoinjury, making it a relevant model for testing cryopreservation strategies (Nishijima *et al.,* 2021). In contrast, dogs are frequently used in reproductive veterinary research and share key physiological features with wild canids, supporting their use in translational studies (Martins *et al.,* 2012). Although neither species is endangered, both offer practical and biologically informative models for evaluating epididymal sperm cryopreservation protocols, particularly in scenarios where ejaculation is not feasible, such as post-mortem recovery or clinical orchiectomy.

Different semen collection methods are used in dogs and rabbits, depending on the physiology and the purpose of the study. In dogs, the most common technique is digital manipulation, where ejaculation is achieved through manual stimulation of the penis. This method often requires the presence of a female in estrus to provide adequate sexual stimulation (Peña & Linde-Forsberg, 2000). In rabbits, the most effective and widely used

method of sperm collection is the use of an artificial vagina to simulate the conditions of the female's reproductive tract. However, this method usually requires a trained male and the presence of a receptive female to stimulate the male (Morrell, 1995; International Rabbit Reproduction Group, 2005). An alternative to traditional methods is sperm retrieval from the cauda epididymis and vas deferens. In clinical and research settings where ejaculation is not feasible—such as elective orchiectomy, sudden death, or wildlife conservation—mature spermatozoa can be retrieved directly from the cauda epididymis, a natural reservoir of functionally competent cells. This region stores mature spermatozoa, and various retrieval techniques allow its use for assisted reproduction (Marks *et al.,* 1994; Luvoni & Morselli, 2017). Despite their lower cryotolerance compared to ejaculated sperm, frozen epididymal spermatozoa have resulted in viable offspring following artificial insemination (Luvoni & Morselli, 2017; Galarza *et al.,* 2021). In rabbits, cauda epididymal sperm demonstrated significantly higher in vitro fertilization rates compared to ejaculated or more proximal epididymal sperm, highlighting its advanced functional maturity (Brackett *et al.,* 1978). Nevertheless, seminal plasma proteins play an essential role in membrane stabilization and capacitation (Bezerra *et al.,* 2019).

Epididymal sperm retrieval techniques are widely used in veterinary practice (Marks *et al.,* 1994; Hewitt *et al.,* 2001). In dogs, epididymal spermatozoa can be retrieved through various methods, including mincing, retrograde flushing, and aspiration. Mincing is simple and yields high sperm counts but may lead to blood contamination. Retrograde flushing provides cleaner samples with better post-thaw motility, while aspiration offers minimal contamination but typically lower sperm yield. Despite their differences, all methods are suitable for cryopreservation and have been used successfully in reproductive programs (Martinez-Pastor *et al.,* 2006; Hori *et al.,* 2015; Ali Hassan *et al.,* 2023). In rabbits, epididymal sperm is retrieved using the retrograde flushing technique (Laghouati *et al.,* 2021). This method is simple, minimizes tissue damage, and allows the preparation of pooled sperm samples for uniform experimental evaluation. An alternative technique involves cannulation of the vas deferens combined with low electrical stimulation of smooth muscle to recover epididymal fluid in a controlled and sterile manner (Jones & Glover, 1973).

Epididymal spermatozoa can be used either fresh or cryopreserved. Fresh samples are suitable for immediate artificial insemination, while frozen-thawed sperm is essential for long-term storage and assisted reproductive technologies (Luvoni & Morselli, 2017).

Compared to ejaculated sperm, epididymal spermatozoa are generally more susceptible to cryoinjury due to morphological and physiological differences, particularly the lack of seminal plasma exposure (Mogheiseh *et al.,* 2022). Despite these limitations, successful fertilizations and births have been achieved using frozen epididymal spermatozoa in dogs, confirming its relevance in reproductive biotechnology (Hori *et al.,* 2004; Luvoni & Morselli, 2017).

Various extenders have been tested for the cryopreservation of epididymal spermatozoa, showing considerable variation in post-thaw motility, membrane integrity, and acrosome preservation. In dogs, commonly used extenders include TRIS-based media supplemented with egg yolk, low-density lipoproteins, or glycerol, as well as several commercial products (Luvoni & Morselli, 2017; Mogheiseh *et al.,* 2022). These extenders aim to protect spermatozoa from cold shock and oxidative stress, yet individual variability among dogs significantly affects freezability, with some samples exhibiting substantial post-thaw declines in viability and acrosomal integrity. In contrast, the cryopreservation of rabbit epididymal sperm remains less studied compared to ejaculated samples. Recent investigations have highlighted the potential of TRIS-based extenders, although the influence of buffer concentration and composition on sperm cryosurvival is still not fully understood (Laghouati *et al.,* 2021).

The thawing temperature is also a key factor affecting sperm viability after cryopreservation. In rabbits, thawing at lower temperatures has been shown to preserve sperm motility better than at higher temperatures (Chen & Foote, 1994). In contrast, some studies in dogs show that thawing at 70°C can improve post-thaw sperm motility and reduce acrosome damage compared to slower thawing at 37°C (Nöthling & Shuttleworth, 2005). However, recent data indicate that different thawing time and temperature in dog sperm did not significantly affect motility (Ibrahim *et al.,* 2024). These findings highlight the species-specific and method-dependent nature of thawing protocols. Further studies are needed to determine the optimal conditions specifically for epididymal spermatozoa.

While TRIS-based extenders and various thawing temperatures have been individually evaluated in prior studies, to our knowledge, no study has systematically evaluated their combined effects on the cryosurvival of epididymal spermatozoa in dogs and rabbits. Therefore, the objective of this study was to assess the influence of two TRIS-based extenders

(198 and 248 mM) and two thawing protocols (37 °C for 30 seconds and 70 °C for 8 seconds) on post-thaw sperm quality in both species, using retrograde flushing as the sperm retrieval method.

2. MATERIAL AND METHODS

2.1. Animals

This study included 22 male dogs *(Canis lupus familiaris)* and 10 rabbits *(Oryctolagus cuniculus)* underwent routine castration at the Veterinary Clinical Hospital (University of Las Palmas de Gran Canaria) during 2024 and 2025. The dog population (Table 1) included 15 mixed-breed dogs and 7 purebred animals; the animals ranged in age from 8 months to 10 years, with an even distribution between those younger and older than one year (n = 11 per subgroup); body weights ranged from 5 to 35 kg. Regarding rabbits, breeds involved were Californian breed, with an age range between 9-12 months and weighting 1.5 and 2.0 kg. Before undergoing surgery, all animals were subject to a thorough physical examination, which included heart rate, glucose levels, rectal temperature, breathing frequency, and mucus color evaluation, as well as a complete blood analysis. The study was carried out in accordance with Spanish legislation on animal protection (Law 7/2023, 28 March 2023) and European Directive 2010/63/EU on the protection of animals used for scientific purposes. As the procedures were performed during routine clinical practice and no additional intervention was conducted beyond standard veterinary surgical protocols, ethical approval was not required according to institutional guidelines.

Table 1. Breed distribution and age range of the dogs included in the study.

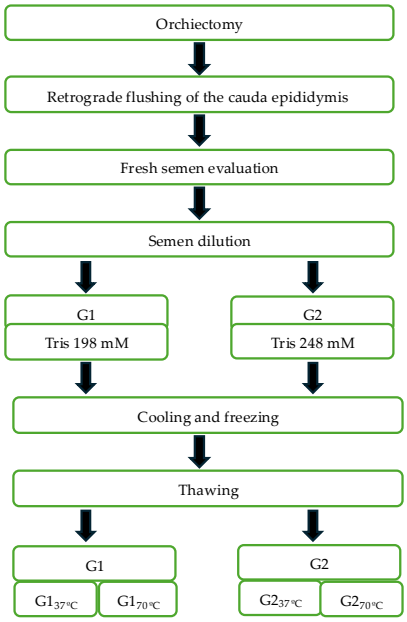
Breed	Animals	Age
Mixed-breed	15	8 months – 10 years
German Shepherd	1	4
Poodle	1	7
Pinscher	1	3
English Setter	1	6
American Bully	1	1
Bulldog	1	6
Rough Collie	1	3

2.2. Experimental design

The study was structured to evaluate the effects of two TRIS-based extenders (198 mM and 248 mM) and two thawing protocols (37 °C for 30 seconds and 70 °C for 8 seconds) on the post-thaw quality of epididymal spermatozoa in two species: dogs and rabbits. Each se-men

sample was divided into four aliquots and assigned to one of the four experimental groups ($G1_{37}$, $G1_{70}$, $G2_{37}$, $G2_{70}$). Sperm quality was evaluated in fresh samples and re-assessed after cryopreservation and thawing. The experimental workflow is summarized in Figure 1.

Figure 1. Experimental design applied to each animal



2.3. Sample collection

Orchidectomy was performed by a standard procedure using a pre-scrotal approach in dogs. Briefly, the skin and subcutaneous tissue were incised (4–8 cm long) and the testicular tunics were opened to reach the spermatic cord and then a double suture was per-formed. In rabbits, a scrotal approach was used (1-2 cm long), and blood vessels and deferent ducts were ligated. For anesthesia, all dogs received similar premedication with Methadone (0,2 mg/kg; Semfortan 10 mg/ml, Dechra Veterinary Products S.L.U., Barcelona, Spain) and Dexmedetomine (0,05 mg/kg; Dexdomitor 0,5 mg, Ecuphar, Barcelona, Spain), and induction was performed using propofol (4 mg/kg IV; Propovet, 10 mg/mL, Esteve, Spain) and anesthesia was maintained with sevoflurane (2%) in 100 % oxygen (1–2 L/min) using mechanical ventilation. For rabbits, a combination of ketamine (15 mg/kg SC; Anesketin 100 mg/ml, Dechra Veterinary Products S.L.U., Barcelona, Spain), midazolam (0.5 mg/kg SC; Midazolam B.Braun, Barcelona) was applied and, about 5 minutes later buprenorphine (0.05 mg/kg; bupredine 0,5 mg/kg, Dechra Veterinary Products S.L.U., Barcelona, Spain) was IV administered and then, anesthesia was induced and maintained with sevoflurane with mask (2-3%) in 100% oxygen. Surgery lasted between 15 and 20 minutes in dogs and between 10 and 15 minutes in rabbits.

Immediately after castration, the testes were transported to the laboratory in sterile containers at room temperature. Epididymal spermatozoa were retrieved using a retro-grade flushing technique, briefly, within 15 minutes after testicular collection, the cauda epididymides, along with 3–4 cm of the proximal vas deferens, were carefully dissected free from the testes, and the surrounding connective tissue and blood vessels were re-moved. After that, a sterile 1 mL plastic syringe with an attached needle was inserted into the vas deferens, and the cauda epididymis was flushed with 1–2 mL of a pre-warmed TRIS-glucose-citrate solution (37°C). The resulting suspension was collected in a sterile Petri dish for further processing and an aliquot of sperm samples were evaluated for total and progressive motility, vigor, viability, abnormal morphology, and acrosome integrity.

2.4. Fresh sperm analysis

The assessment of total and progressive sperm motility was performed using phase-contrast microscopy with a heated stage set at 37°C. Total motility was subjectively estimated by evaluating at least ten microscopic fields at 200× magnification and recorded to the nearest 5%. Progressive motility was assessed based on the percentage of spermatozoa moving in a forward, linear direction (Hewitt *et al*., 2001). Sperm vigor was evaluated using a subjective scoring system based on the intensity and pattern of movement under phase-contrast microscopy. The viability of spermatozoa and the percentage of abnormal sperm cells were assessed using Eosin–Nigrosin stain, following Christiansen's classification. Between 100 and 200 spermatozoa were evaluated under phase-contrast microscopy at 1,000× magnification. Sperm morphology was classified as normal or abnormal based on structural defects observed in the head (e.g., pyriform shape, double heads, detached heads), midpiece (e.g., bent, irregular thickness), and tail (e.g., coiled, multiple tails, distal droplets). Spermatozoa with retained proximal or distal cytoplasmic droplets were categorized as immature forms. Acrosome integrity was evaluated using the Diff-Quick staining technique (Mazzuchini *et al.,* 2024). with at least 100 cells analyzed under phase-contrast microscopy at 1,000× magnification. All assessments were performed by the same trained operator to reduce inter-observer variability.

2.5. Diluent preparation and sperm processing

All samples were processed individually. After the initial evaluation, sperm samples were centrifuged at 700 × g for 8 minutes at room temperature (20–22°C). Each sample was then divided into two aliquots and diluted to reach a final sperm concentration of 100–200 × 10^6

spermatozoa/mL and samples were diluted with two different extenders: G1 (Ext-1 group) and G2 (Ext-2 group); the compositions of extenders are described in Table 2.

Table 2. Composition of extenders used for cryopreservation

Component	Ext-1a (G1, 1st step)	Ext-1b (G1, 2nd step)	Ext-2a (G2, 1st step)	Ext-2b (G2, 2nd step)
TRIS	198 mM	198 mM	248 mM	248mM
Citric Acid	73 mM	73 mM	73 mM	73 mM
Glucose	44 mM	44 mM	44 mM	44 mM
Cefazolin	0.1 g	0.1 g	0.1 g	0.1 g
Egg yolk	20%	20%	20%	20%
Glycerol	3%	7%	3%	7%

The diluted samples were gradually cooled to 4 °C over a period of approximately 1 hour by placing the tubes in a temperature-controlled laboratory refrigerator (range 4 ± 1 °C). The cooling process was monitored using a digital thermometer inserted in a control tube containing extender only, allowing verification of the desired temperature profile. Then a second extender solution was added in a volume equal to the first extender: Ext-1b was added to samples initially diluted with Ext-1a; Ext-2b was added to samples initially diluted with Ext-2a, to result in a final sperm concentration of 50–100 × 10^6 spermatozoa/mL. Immediately after adding the second extender, an aliquot was taken to evaluate sperm motility and confirm proper temperature adaptation. Approximately 10 minutes later, the samples were loaded into 0.5 mL plastic straws at 4 °C and placed horizontally on a rack positioned 4 cm above the liquid nitrogen level in a foam box for 10 minutes, before being fully submerged for final freezing and storage.

2.6. Thawing and sperm analysis

Frozen sperm samples were thawed after freezing using a programmable water bath. Two different thawing protocols were used: 37°C for 30 seconds and 70°C for 8 seconds and, therefore, 4 different groups resulted: G137, G170, G237 and G270. After thawing, the contents of each straw were transferred to plastic tubes, and sperm samples were first maintained at room temperature for 5 minutes, followed by incubation at 37°C for 10–12 minutes. Post-thaw sperm quality was evaluated using the same methods as for fresh samples, including: total and progressive motility, vigor, viability (Eosin-Nigrosin stain), acrosome integrity (Diff-Quick stain). For experimental group, in dogs, at least one aliquot per sample

was analyzed, while in rabbits, between 2-3 samples were assessed in each experimental group to compensate for the lower number of rabbits in the study.

2.7 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 29.0 (IBM Corp., Armonk, NY, USA). Prior to conducting inferential tests, the assumptions of normal distribution and homogeneity of variances were verified. Normality of continuous variables (motility, vigor, viability, and acrosome integrity) was assessed using the Shapiro-Wilk test ($p > 0.05$ for all variables), and homoscedasticity was tested with Levene's test. No data transformations were required. Continuous data were expressed as mean ± standard deviation (SD), while categorical data (presence of specific sperm abnormalities) were presented as frequencies and percentages. A univariate analysis of variance (ANOVA) was conducted using the General Linear Model (GLM) procedure. The model included three fixed factors: donor age (<1 year vs. >1 year), type of cooling extender (G1 vs. G2), and thawing condition (37 ºC for 30 seconds vs. 70 ºC for 8 seconds), as well as their two-way and three-way interactions. Male identity was included as a random effect to account for repeated measures. When significant differences were detected, Duncan's multiple range test was used for post hoc comparisons. This test was selected for its sensitivity in detecting potential differences in exploratory research. A p-value < 0.05 was considered statistically significant.

3. RESULTS

The flushing process was performed without any difficulty in medium and large size dogs, as their larger testicles facilitated easier flushing and a higher volume of the washing solution (WS) was used; in small dogs the limited size of the epididymis and vas deferens reduced the volume of WS that could be effectively used for sperm retrieval. In rabbits, testicular size (comparable to that of small dogs) was consistent across individuals, which contributed to a more standardized and uniform flushing procedure with no noticeable differences in handling or technical difficulty. About 60% of samples in both species did not contain the blood or parts of foreign tissues.

3.1. Dogs

Table 3 shows the mean values of main parameters of canine sperm samples immediately after collection. Regarding age, the mean values of all parameters were close, with no significant differences between groups of animals younger and older than one year. In total,

the samples showed high total and progressive motility, with a mean vigor score higher than 3.5 indicating strong and consistent movement among spermatozoa. Viability and acrosome integrity were also high, reflecting good sperm quality. As for individual values, acrosome integrity values were found to be relatively uniform across individuals, but parameters such as viability or progressive motility showed considerable individual variation.

Mean percentages of total sperm abnormalities and their specific types (head, middle piece, tail) in fresh canine sperm are shown in Table 4. Regarding age, no significant differences were observed between groups of animals, although dogs older than one year exhibited a higher proportion of head abnormalities, whereas younger dogs had a greater incidence of immature spermatozoa characterized by cytoplasmic droplets. In total, the highest proportion of abnormalities (approximately 60%, $p<0.01$) was associated with immature sperm cells, followed by tail abnormalities, while between head and middle piece abnormalities were not significantly differences. As for individual values, the number of abnormal forms showed considerable individual variation, especially in the per-centage of immature cells.

Table 3. Mean (± sd) and range of sperm motility, vigor, viability and acrosome integrity in the fresh sperm of dogs

Age (Years)	Fresh sperm features				
	Total motility %	Progressive motility, %	Vigor (0-5)	Viability %	Acrosome integrity %
< 1 year	75.50 ± 4.19	59.50 ± 3.72	3.60 ± 0.23	84.90 ± 2.66	90.30 ± 1.17
Range	50-90	35-70	2-4	68-94	85-95
> 1 year	77.50 ± 3.62	61.00 ± 3.58	3.90 ± 0.10	86.90 ± 1.99	91.80 ± 0.78
Range	60-90	45-75	3-4	72-93	86-94
Total	76.82 ± 2.39	60.45 ± 2.23	3.77 ± 0.11	86.32 ± 1.48	91.00 ± 0.63
Range	50-90	35-75	2-4	68-94	85-95

Table 4. Sperm abnormalities (head, middle piece, tail) and immature sperm cells in fresh sperm of dogs (mean ± sd)

Age (Years)	Total abnormalities	Head	Middle piece	Tail	Immature sperm cells
< 1 year	26.90 ± 2.03	0.6^{a} ± 0.23	0.70^{a} ± 0.35	7.60^{b} ± 0.65	17.90^{c} ± 1.79
Range	20-40	0-2	0-3	5-12	12-30
> 1 year	23.90 ± 2.20	1.70^{a} ± 0.61	1.30^{a} ± 0.45	6.80^{b} ± 0.56	14.20^{c} ± 1.70
Range	14-33	0-5	0-4	4-9	7-22
Total	25.13 ± 1.34	1.23^{a} ± 0.31	1.05^{a} ± 0.26	7.32^{b} ± 0.45	15.54^{c} ± 1.19
Range	14-40	0-5	0-4	4-12	7-30

^{abc}; Different letters in the same file denote significant differences ($p<0.01$)

Table 5 presents the post-thaw sperm parameters across all experimental groups of dogs. In all groups, a significantly decrease in motility ($p<0.01$) was evident with respect to the values in fresh sperm. Higher values of total and progressive motility were observed in samples from groups G170 and G270, with mean values nearly twice ($p<0.01$) as high as those recorded in groups G137 and G237. However, no significant differences were found in vigor among any of the groups. Regarding individual variability, a similar pattern was observed across all groups, as reflected in the wide range of values recorded for both total and progressive motility within each group. With respect to the influence of temperature, no significant differences in motility were found within the same extender group (G137 vs. G170 and G237 vs. G270), although samples thawed at 70 °C (G170 and G270) tended to show higher values.

Viability and acrosome integrity in post-thaw sperm are shown in Table 6. In post-thaw viability, with mean values between 61.90 and 66.38%, no significant differences were found between groups. A similar trend was observed in acrosome integrity, where no relevant differences were found between groups, with mean values around 70-75%. No significant differences in viability and acrosome integrity were found between thawing temperatures groups within the same extender group. Similarly to motility, individual variability was evident in all experimental groups, as reflected in the wide range of values observed in both viability and acrosome integrity.

Table 5. Mean (± sd) and range of total, progressive motility and vigor in fresh and post-thaw sperm of the experimental groups of dogs

	Fresh	$G1_{37}$	$G2_{37}$	$G1_{70}$	$G2_{70}$
Total motility (%)	76.82 ± 2.39	22.80^{a} ± 1.77	24.51^{a} ± 1.72	37.54^{b} ± 1.87	46.70^{c} ± 1.77
Range	50-90	10-40	10-40	20-50	30-60
Progressive motility (%)	61.14 ± 2.40	11.36^{a} ±1.13	12.87^{a} ± 1.00	22.95^{b} ± 1.48	30.45^{b} ± 1.71
Range	35-75	5-20	5-25	10-35	15-45
Vigor (0-5)	3.77 ± 0.11	2.04 ± 0.10	2.57 ± 0.11	2.41 ± 0.11	2.95 ± 0.08
Range	2-4	1-3	1-3	2-3	2-4

^{abc}; Different letters in the same file denote significant differences ($p<0.01$)

Table 6. Viability and acrosome integrity in fresh and post-thaw sperm of the experimental groups of dogs (mean ± sd)

	Fresh	$G1_{37}$	$G2_{37}$	$G1_{70}$	$G2_{70}$
Viability (%)	86.32 ± 1.48	61.90 ± 2.08	65.45 ± 1.80	63.79 ± 1.93	66.38 ± 1.77
Range	68-94	35-80	40-80	40-76	40-75
Acrosome integrity (%)	91.00 ± 0.63	71.94 ± 1.13	73.37 ± 1.00	70.43 ± 1.23	70.09 ± 0.95
Range	85-95	60-80	59-79	55-78	60-77

3.2. Rabbits

Table 7 shows the mean values of the parameters of fresh rabbit sperm. The samples exhibited high total motility and moderate progressive motility, with vigor scores ranging between 3 and 5, indicating a generally strong movement pattern across samples. Viability and acrosome integrity were also high, reflecting good sperm quality in fresh samples. Individual variability was observed among animals, in all sperm parameters assessed. The percentages and type of morphological abnormalities are shown in Table 8; similar to dog sperm, approximately 50% of total number of abnormalities was associated with immature sperm cells. However, unlike dogs, tail abnormalities showed mean values ($p>0.05$) close to those of immature sperm cells.

Table 7. Mean (± sd) values of sperm motility, vigor, viability, acrosome integrity and the percentage of abnormalities in the fresh sperm of rabbits

	Fresh sperm features				
	Total motility (%)	Progressive motility (%)	Vigor (0-5)	Viability (%)	Acrosome integrity (%)
Mean	79.00 ± 3.91	52.00 ± 4.17	4.20 ± 0.21	86.70 ± 2.27	83.10 ± 1.81
Range	60-90	35-75	3-5	74-91	10-35

Table 8. Sperm abnormalities (head, middle piece, tail) and immature sperm cells in fresh samples of rabbits (mean ± sd)

	Total abnormalities	Head	Middle piece	Tail	Immature sperm cells
Mean	20.60 ± 2.45	1.50^{a} ± 0.45	1.20^{a} ± 0.41	7.60^{b} ± 1.33	10.30^{b} ± 1.78
Range	10-35	0-4	0-3	2-14	4-19

[ab]; Different letters in the same file denote significant differences ($p<0.01$)

Table 9 summarizes the mean values of motility and vigor in post-thaw sperm samples from rabbits. The best preservation of sperm motility and vigor was observed in G237. In contrast, G170 showed the lowest values in all categories, particularly in total motility (< 5%). Individual variability was evident across all groups, as indicated by the wide ranges observed for motility and vigor (e.g., total motility ranging from 0 to 40%). Regarding the thawing temperature, the highest percentage of motility was observed at a thawing temperature of 37°C, with significant differences between the groups.

Table 9. Mean (± sd) and range of total, progressive motility and vigor in fresh and post-thaw sperm of the experimental groups of rabbits

	Fresh	$G1_{37}$	$G2_{37}$	$G1_{70}$	$G2_{70}$
Total motility (%)	79.00 ± 3.91	13.75^{a} ± 2.37	29.00^{b} ± 3.49	4.92^{c} ± 1,51	13.67^{a} ± 1.96
Range	60-95	0-21.7	5-40	0-10	0-20
Progressive motility (%)	52.00 ± 4.17	6.00^{a} ± 1.10	14.08^{b} ± 1.58	2.33^{a} ± 0.74	7.20^{a} ± 1.01
Range	35-75	0-10	5-20	0-5	0-10
Vigor (0-5)	4.20 ± 0.21	1.80^{a} ± 0.26	2.28^{a} ± 0.21	0.97^{b} ± 0.30	1.87^{a}± 0.25
Range	3-5	0-3	1-3	0-2	0- 2.7

[abc]; Different letters in the same file denote significant differences ($p<0.01$)

Table 10 presents the mean values of viability and acrosome integrity in post-thaw rabbit sperm samples. Among the post-thaw groups, viability was slightly higher in the G237 group, but without significant differences with the G137. No significant differences in acrosome integrity were observed between extender and temperature groups, with close mean values. Individual variability was evident across all groups, as indicated by the broad range of values observed, especially in G270 (viability: 20-60%).

Table 10. Mean (± sd) of viability and acrosome integrity in fresh and post-thaw sperm of the experimental groups of rabbits

	Fresh	$G1_{37}$	$G2_{37}$	$G1_{70}$	$G2_{70}$
Viability (%)	86.70 ± 2.27	52.28 ± 3.59[a]	54.97 ± 3.28[a]	42.92 ± 2.88[b]	47.55 ± 3.87[ab]
Range	74-95	30-62.7	35-67.3	27-55.7	20-60
Acrosome integrity (%)	83.10 ± 1.81	43.10 ± 3.91	48.18 ± 3.41	44.43 ± 3.72	42.88 ± 3.23
Range	74-91	26-60.7	28-65	22-59	23-59.3

[ab]; Different letters in the same file denote significant differences ($p<0.05$)

4. DISCUSSION

In this study, the quality of fresh and post-thaw sperm of dogs and rabbits obtained from the epididymis by flushing method was evaluated. We compared the effects of two TRIS concentrations and two thawing temperatures on post-thaw sperm quality in both species, selecting these conditions based on prior studies and their relevance to reproductive practice. Nevertheless, future research should investigate a broader range of TRIS levels and thawing conditions to refine species-specific cryopreservation protocols and further improve post-thaw outcomes. Epididymal sperm cryopreservation has been extensively studied in dogs. However, data on post-thaw sperm quality remain limited. To our knowledge, no studies have comprehensively evaluated post-thaw epididymal sperm quality in rabbits following retrograde flushing.

In dogs, the fresh sperm samples showed high-quality characteristics, which is consistent with previous findings demonstrating the high functional competence of cauda epididymal spermatozoa (Hori *et al.,* 2004; Martins *et al.,* 2012). Although epididymal spermatozoa are not exposed to seminal plasma, their structural integrity and motility potential remain high when samples are collected immediately after castration and processed under controlled conditions (Hori *et al.,* 2005; Ponglowhapan & Chatdarong, 2008; Korochkina *et al.,* 2014). In our study, motility, vigor, and viability showed consistently high values across fresh samples, with minimal individual variation. This suggests that sperm cells stored in the cauda epididymis are already metabolically and functionally competent for fertilization (Yu & Leibo, 2002). The proportion of intact acrosomes in our study was comparable to previous studies (Hori *et al.,* 2015), supporting the notion that timely collection and processing under controlled conditions allow epididymal spermatozoa to preserve acrosomal integrity until cryopreservation. However, our values were slightly higher than those reported in some

earlier studies (Varesi *et al.,* 2014; Prapaiwan *et al.,* 2016), which may be explained by shorter processing time and reduced mechanical stress during handling. Regarding morphological characteristics, a relatively high proportion of immature spermatozoa was observed in fresh samples, a common feature in epididymal sperm, and is typically associated with cytoplasmatic droplets, which indicate incomplete maturation (Varesi *et al.,* 2013). Hori *et al.* (2005) reported that spermatozoa collected directly from the cauda epididymis commonly exhibit cytoplasmic droplets and suggested that exposure to prostatic fluid during ejaculation facilitates their removal. This supports the hypothesis that sperm maturation continues at the moment of ejaculation in dogs. Our findings also align with the report by Hori *et al.* (2011), who observed that cryopreserved cauda epididymal sperm in dogs, when incubated with prostatic fluid, exhibited comparable post-thaw motility and viability to ejaculated sperm. Moreover, in intrauterine insemination trials, conception rates were similarly low between epididymal and ejaculated sperm, suggesting no significant difference in fertilization potential between the two sources (Hori *et al.,* 2011).

The analysis of sperm quality in dogs grouped by age (<1 year and >1 year) showed comparable mean values across evaluated parameters, suggesting that overall sperm functionality remained stable after retrieval from the cauda epididymis. However, dogs > 1 year exhibited a slightly higher percentage of head abnormalities, while younger dogs showed a greater proportion of immature spermatozoa. Although the number of animals in each group was limited, and these trends were not statistically significant, they may reflect ongoing dynamics of sperm maturation in younger individuals and potential early signs of degenerative changes in older males. These observations are consistent with previous studies on fresh semen, which describe a relationship between age and sperm morphology. Tesi *et al.* (2018) found that older dogs tend to exhibit a higher percentage of structural abnormalities, while younger animals may show a greater presence of immature sperm forms. Moreover, Lechner *et al.* (2022) noted that while sperm motility remains relatively stable until advanced age, minor morphological alterations may begin to appear earlier, which could negatively impact cryotolerance. In addition, Zmudzinskaya *et al*. (2022) reported a significant decrease in total and progressive motility in older dogs. These trends are consistent with literature indicating that aging may lead to morphological changes (Tesi *et al.,* 2018; Lechner *et al.,* 2022) and reduced motility (Zmudzinska *et al.,* 2022). While dogs of different breeds were included in the study, the limited sample size per breed did not allow for meaningful statistical comparisons. Rather than stratifying by breed with insufficient power, we

prioritized maintaining analytical consistency across the entire dataset. Nevertheless, this diversity reflects real-world clinical conditions and highlights the need for future studies with larger, breed-stratified cohorts to determine whether specific genetic backgrounds influence epididymal sperm quality and cryotolerance. Such investigations would be valuable for refining breed-specific semen preservation protocols and improving outcomes in canine reproductive biobanking.

Rabbit fresh epididymal sperm, similar to dogs, showed high overall quality across evaluated parameters, Acrosome integrity and viability showed stable values across samples, indicating effective epididymal maturation. Our results were consistent with those reported by Iaffaldano *et al.* (2012) about ejaculated rabbit sperm. The high initial values observed confirm that cauda epididymal sperm maintains structural and functional integrity when retrieved immediately after castration (Gloria *et al.,* 2021). Our findings are consistent to those reported by Brackett *et al.* (1978), who demonstrated that cauda epididymal sperm had a high ability to fertilize in vitro, supporting the idea that epididymal maturation is crucial for acquiring fertilizing capacity in rabbits. Despite the overall good quality of our samples, some variability was noted, particularly in the percentage of morphologically abnormal cells. Bezerra *et al.* (2019) reported a high percentage of head defects and a low number of cells with cytoplasmatic droplets in ejaculated rabbit semen. In contrast, our results showed a low proportion of head abnormalities, while the percentage of immature spermatozoa was relatively high, as described in dogs.

In dogs, post-thaw evaluation of epididymal spermatozoa revealed a reduction in quality parameters compared to fresh samples. Nevertheless, some quality parameters remained at high levels, with viability exceeding 60% and a high proportion of intact acrosomes. These findings are consistent with previous studies reporting variable post-thaw sperm quality depending on freezing protocols and extender composition (Martins *et al.,* 2012; Prapaiwan *et al.,* 2016). In our study, total and progressive motility were higher in samples thawed at 70 °C for 8 seconds, regardless of the extender used. These findings are consistent with previous studies on ejaculated dog semen, which reported that thawing at 70 °C for 8 seconds resulted in significantly improved motility, viability, and acrosome integrity compared to thawing at 37 °C for 15 seconds (Peña & Linde-Forsberg, 2000; Nöthling & Shuttleworth, 2005). On the other hand, Brito *et al.* (2017) reported no significant improvement in motility when comparing fast and slow thawing protocols for ejaculated semen. Similarly, Ibrahim *et al.*

(2024) found no significant differences across thawing temperatures, but noted that fast thawing performed comparably to slow thawing, suggesting that higher temperatures may be safe and even beneficial under optimized conditions. Despite the overall good preservation in dogs, individual variability was evident, particularly in motility and viability and some samples showed total motility as low as 5%, suggesting that, even under standardized conditions, cryosurvival capacity may differ markedly between individuals. This observation aligns with previous findings that highlight the influence of factors such as donor age, health status, and initial sperm quality on freezability of both epididymal (Zmudzinska *et al.,* 2022) and ejaculated spermatozoa (Rijsselaere *et al.,* 2007; Tesi *et al.,* 2018).

In dogs, previous studies using 198 mM TRIS-based extenders reported post-thaw motility values ranging from 24% to 45% in canine epididymal sperm (Martins *et al.,* 2012; Batista *et al.,* 2016), which are comparable to the results obtained with Extender-1. However, our Extender-2 group showed improved motility without negatively affecting other functional parameters. Although the differences were not statistically significant, the observed trend suggests that increasing the TRIS concentration may enhance sperm protection during cryopreservation. This effect may be particularly relevant for epididymal spermatozoa, which lack exposure to seminal plasma components known to contribute to membrane stabilization (Prapaiwan *et al.,* 2016). Therefore, while a concentration of 198 mM TRIS remains a well-established standard, 248 mM may provide additional protective effect, likely due to its greater buffering capacity and improved osmotic balance during the freezing and thawing processes. This supports the idea that a higher TRIS concentration provides better buffering capacity and protection against cryoinjury. (Hori *et al.,* 2011; Prapaiwan *et al.,* 2016).

Rabbit sperm demonstrated a marked decrease in all assessed parameters after cryopreservation. This significant reduction reflects the greater sensitivity of rabbit spermatozoa to cryoinjury, which may be attributed in part to the elongated structure of the rabbit sperm head making them more susceptible to physical damage during freezing and cold shock (Mahadevan & Trounson, 1984; Isachenko *et al.,* 2011). These findings are consistent with those by Gloria *et al.* (2021), who demonstrated that rabbit sperm motility is sensitive to osmotic changes, with a more rapid decrease in motility than in membrane integrity when exposed to non-physiological extenders and temperature fluctuations. Kubovičová *et al.* (2022) also highlighted some characteristics of rabbit sperm, including low water

permeability and a high activation energy for water transport, which makes them particularly susceptible to intracellular ice formation and osmotic imbalance during freezing and thawing.

In rabbits, no significant differences were observed in viability and acrosome integrity, despite the extender and the thawing temperature used. However, samples frozen with 248 mM TRIS showed slightly better motility, especially at 37 °C. Previous studies supported the use of TRIS-based extenders for rabbit semen preservation. For instance, Di Iorio *et al.* (2020) reported favorable outcomes using an extender containing 250 mM TRIS, and Laghouati *et al.* (2023) demonstrated good motility with 310 mM TRIS supplemented with natural antioxidants. These variations highlight the absence of a clearly defined optimal TRIS concentration for rabbit sperm preservation. Nevertheless, our findings suggest that a slightly elevated TRIS concentration (closer to 250 mM) may enhance sperm protection during freezing, likely due to improved buffering capacity and osmotic stability.

Individual variability among rabbits was pronounced, with some samples exhibiting a complete loss of motility after thawing. In contrast, although some canine samples showed reduced motility (as low as 5%), none dropped to zero, suggesting a generally higher cryotolerance of canine epididymal spermatozoa. These findings highlight marked interspecies differences in sperm sensitivity to cryoinjury, likely reflecting a combination of intrinsic cellular characteristics—such as membrane lipid composition, osmotic tolerance, and metabolic stability—and the degree to which the cryopreservation protocol is suited to each species. In rabbits, the limited post-thaw motility may be partially attributed to a suboptimal match between extender composition, cooling/freezing rates, and thawing temperature. Notably, our results showed that thawing at 37 °C yielded better motility outcomes in rabbits, corroborating previous reports indicating that moderate thawing temperatures (<50 °C) are more appropriate for this species. This underscores the importance of species-specific optimization of cryopreservation protocols. (Mocé *et al.,* 2003; Mogheiseh *et al.,* 2022).

Due to the limited number of studies specifically addressing the cryopreservation and post-thaw evaluation of rabbit epididymal spermatozoa, interpreting our findings presents a challenge. Although some general principles of sperm maturation and cryobiology are shared across mammalian species, species-specific differences in epididymal physiology and cryotolerance must be considered. Our results therefore contribute to narrowing this knowledge gap by providing original data on the functional characteristics of rabbit

epididymal sperm before and after freezing, which may serve as a reference for future reproductive and biobanking applications in this species (Ali Hassan *et al.,* 2021). In contrast to rabbit spermatozoa, which showed a marked decline in post-thaw motility in our study, epididymal sperm from other species—such as domestic cats—has demonstrated comparatively better cryosurvival outcomes (Kunkitti *et al.,* 2016). These findings underscore the need to optimize cryopreservation protocols tailored to the unique physiology of each species. In particular, the interaction between TRIS concentration and other extender components warrants further investigation, especially in epididymal spermatozoa, which may respond differently from ejaculated counterparts.

The relevance of this study is further underscored by the practical applications of epididymal spermatozoa in reproductive biotechnology. The use of rabbits as a model species in this study is supported by their recognized value in both agricultural and biomedical fields (Nishijima *et al.,* 2021). Rabbits are not only an important source of meat and fur but are also extensively used in biomedical research, particularly for antibody production and as experimental models for cardiovascular and metabolic diseases (Nishijima *et al.,* 2021). This context underscores the importance of optimizing rabbit sperm cryopreservation protocols, including the study of epididymal spermatozoa. In dogs, epididymal sperm is an essential resource for assisted reproductive technologies—especially when collected post-mortem or after orchiectomy—including artificial insemination. The present findings confirm the value of epididymal sperm retrieval and offer practical implications for cryobank development and assisted reproduction programs in domestic species and potentially, after appropriate species-specific adaptations, in conservation efforts involving wild animals. Nevertheless, further research is needed to optimize extender formulations, improve cryotolerance and better understand the physiological factors influencing sperm quality following castration and cryopreservation.

5. CONCLUSIONS

1. Epididymal sperm obtained by flushing showed good quality in fresh samples from both dogs and rabbits, confirming its potential for reproductive use.
2. In dogs, thawing at 70 °C for 8 seconds and the use of 248 mM TRIS improved post-thaw motility, although no differences were observed in viability or acrosome integrity.
3. In rabbits, sperm quality decreased markedly after cryopreservation, with better outcomes observed when thawed at 37 °C.

4. The higher TRIS concentration also showed a slight improvement in motility in rabbits, although no significant differences were found in other parameters.

5. High individual variability was observed, suggesting that factors such as age or initial semen quality influence cryotolerance.

6. The high proportion of immature spermatozoa is expected in epididymal samples due to the lack of exposure to seminal plasma.

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