



COLLECTION, PRESERVATION, AND ASSESSMENT OF CANINE EPIDIDYMAL SEMEN – CURRENT KNOWLEDGE AND FUTURE CHALLENGES – A REVIEW*

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Abstract

Epididymal spermatozoa have great potential as an alternative source of canine germ cells for the preservation of the unique features of both endangered canid species and purebred dogs. Epididymal semen collected from exceptional individuals is usually cryopreserved and constitutes the final reserve of desired biological material. Cryopreservation of semen recovered from the epididymis poses difficulties related to obtaining samples at sufficient concentrations, in addition to its lower cryotolerance than ejaculated semen. Meeting the challenges associated with the conservation of epididymal sperm necessitates delving deeper into the available methods for its acquisition and preservation, in addition to applying appropriate evaluation methods. The quality of cryopreserved epididymal sperm can be influenced by a number of factors, from the collection technique applied to the insemination process utilized. Furthermore, our review provides a comparison of the effectiveness of selected extenders and their supplements and highlights aspects that require improvements or further investigation. The analyzed literature data show that squeezing and retrograde flushing technique of obtaining semen from the epididymis minimizes tissue and erythrocytes contamination. In order to ensure the best quality of epididymal semen after thawing, storage at 4–5°C should be avoided for longer than 24 h in 0.9% saline or 5 days in chilling extenders. We concluded that the egg yolk based extenders and Bovimix® are the most efficient methods of cryopreservation of canine epididymal sperm, however, the use of supplementation such as antioxidants requires further investigation.

Key words: canine epididymal semen, cryopreservation, sperm quality parameters

The epididymis is the structure in which spermatozoa undergo the process of maturation and storage (Chłopik and Wysokińska, 2020). This organ, located between the efferent ducts of the testis and the vas deferens, is formed by twisted tubules divided into the following anatomical sections: the caput, corpus, and cauda (James et al., 2020). The process of sperm maturation during passage through subsequent structures involves acquiring the ability to move by activating the functions of mitochondria, which is related to the migration of the cytoplasmic droplet (Angrimani et al., 2017). Therefore, only spermatozoa retrieved from the cauda are motile and potentially able to fertilize (Mogheiseh et al., 2022).

However, the fertilizing potential of preserved epididymal semen remains the subject of numerous studies providing different conclusions. The authors of previous research have reported that cryotolerance could be comparable to ejaculated spermatozoa (Hori et

al., 2015, 2011; Mogheiseh et al., 2022) or highlight the fact that the observed low freezing tolerance is caused by morphological immaturity and sperm membrane instability (Ali Hassan et al., 2023; Korochkina et al., 2014; Ponglowhapan and Chatdarong, 2008). Although the possibility of obtaining epididymal semen of good quality and concentration through this method is reduced compared to ejaculated sperm, in special cases, epididymal spermatozoa could be the only available source of valuable gametes. The postmortem recovery of sperm from the epididymis demonstrates potential usefulness in preserving Canidae species biodiversity. Utilization of this method has been described for Arctic fox, gray wolf, African wild dog, maned wolf, and red wolf, with limitations linked to epididymides without sperm during the non-breeding season or the reduced value of sperm from dead, old or immature males (Nagashima and Songsasen, 2021).

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Semen cryopreservation is an important tool in assisted reproduction technologies (ARTs), widely used in canids (Payan-Carreira et al., 2011). Cryopreservation is particularly important in the international exchange of genetic material from purebred dogs with unique phenotypic and functional characteristics (Suzuki et al., 2022; Joonè, 2024). In addition, it allows for the preservation of gametes from endangered species and rare breeds of animals for an unlimited duration (Trzcińska and Samiec, 2021). Of late, research on procedures for the cryopreservation of canine ejaculated semen and its use in ARTs has markedly expanded (Linde-Forsberg, 2001; Ibrahim et al., 2024; Joonè, 2024; Suzuki et al., 2022). However, relatively few studies have been published on practical methods for the freezing and cooling of epididymal semen that could be easily utilized in veterinary practice (Ponglowhapan and Chatdarong, 2008). That is why this article is devoted to the topic of long-term storage of epididymal semen as a method of preserving biological material from valuable male canids.

Through the following review, we aim to present both the collection and freezing processes used for canine epididymal semen and to critically evaluate methods applied for canine sperm analysis.

Methods used for epididymal semen collection

The technique of collecting material from gonads *ex vivo* requires prior dissection of the epididymis from the testicles. *In vivo* collection procedures are used in human andrology as a minimally invasive method to avoid surgery on testicular tissue (Varesi et al., 2013). The methods used for the retrieval of sperm from this organ are shown in Figure 1.

Techniques used for the collection of sperm from *ex vivo* epididymis

Following orchiectomy and before any form of manipulation, testis including the epididymis can be preserved in 0.9% saline solution alone (Wysokińska et al., 2021) or with antibiotic treatment – gentamycin (Simons et al., 2019) or penicillin and streptomycin (Ali Hassan et al., 2023). If it is not possible to obtain material immediately, the testicles may be stored in that solution at 4°C for a maximum of 24 hours (Ali Hassan et al., 2023; Simons et al., 2019); at 15–25°C for a maximum of 20 hours (Nöthling et al., 2007); or at 37°C for a maximum of 2 hours (Ponglowhapan et al., 2006). Thereafter, the testis and epididymis are washed using Dulbecco's phosphate-buffered saline (DPBS) and wiped carefully. A practical tip is to crimp the clamps at the remaining end of the plexus pampiniformis to prevent blood from flowing into the collection area. Subsequently, the ductus deferens and the testicular artery are separated and the latter is removed. The epididymis within the vas deferens should be carefully separated from the testis, and a disposable scalpel blade should be used only for cutting the junction between the testis and cauda (Hori et al., 2015; Simons et al., 2019). The prepared epididymis is placed in a Petri dish with a chosen medium; thereafter, an appropriate method of semen collection is performed.

Various methods for the retrieval of cauda epididymal sperm have been described in the literature, including squeezing, retrograde flushing, mincing (float-up or swim-up method), and epididymal sperm aspiration (Ali Hassan et al., 2023, 2021; Hori et al., 2015; Luvoni and Morselli, 2017).

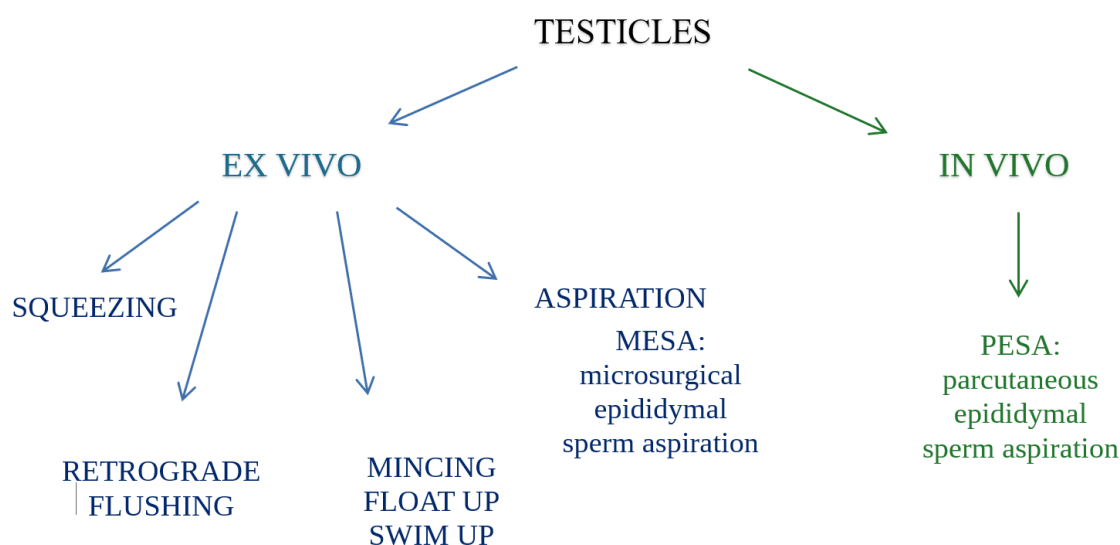


Figure 1. Methods used for the recovery of epididymal sperm

- a) **The squeezing method** requires a filled epididymis, which is emptied using anatomical clamps moved from the caudal region toward the vas deferens (Martins et al., 2009). The limitation of this method is the lower concentration of the obtained sample (Luvoni and Morselli, 2017).
- b) **Retrograde flushing (RF)** requires isolation of the caudal part along with the vas deferens from the remaining segments of the epididymis. Thereafter, a 24-gauge blunt needle is inserted into the proximal section of the vas deferens to wash out the content using 0.5–2.5 ml of medium. This method cannot be used in the case of narrow caudal ductus from small dogs or if the vas deferens is shortened or obstructed (Ali Hassan et al., 2023; Luvoni and Morselli, 2017; Nöthling et al., 2007).
- c) **Epididymal mincing (EM)** can also be referred as the float-up or swim-up method (Ali Hassan et al., 2021; Hori et al., 2004; Klinc et al., 2005; Simons et al., 2019). The analyzed literature shows that the differences in nomenclature may reflect the severity of fragmentation of the epididymal tissue. The procedure can be performed using two different methods:
 - 1 mm slicing (literally chopping or “mincing” the epididymis) (Klinc et al., 2005).
 - Single cutting and incubation for 10 to 30 minutes to facilitate spermatozoa transmigration from the incision to the medium (“float-up” or “swim up”) (Ali Hassan et al., 2021; Hori et al., 2004; Simons et al., 2019). The use of blade 24 (Simons et al., 2019) or 23 (Hori et al., 2015) is recommended.

This method allows for the collection of samples regardless of the size of the epididymis (Ali Hassan et al., 2023; Klinc et al., 2005). Typically, after slicing the cauda, the medium should be filtered using a 40 µm (Hori et al., 2011) or 80 µm (Garcia-Macias et al., 2006; Hewitt et al., 2001) metal mesh filter to remove tissue fragments. One disadvantage of this method is the contamination of the sample with blood (Rijsselaere et al., 2004). Hemoglobin is the source of iron ions, which increase the production of reactive oxygen species, disrupt the balance of antioxidant processes, and enhance lipid peroxidation (Ali Hassan et al., 2021). The protocol of erythrocyte elimination prior to freezing should be implemented when the blood content is more than 4% (Prapaiwan et al., 2016). Gradient centrifugation results in the loss of spermatozoa in ejaculated sperm, which is not permitted in less concentrated epididymal samples (Domain et al., 2022). The addition of erythrocyte lysis buffer to the freezing extender causes lower motility and a higher intracellular ROS content in epididymal spermatozoa (Ali Hassan et al., 2023). Erythrocyte–sperm separation media and microfluidic systems are alternative methods but have not yet been used for canine epididymal semen (Ali Hassan et al., 2021).

For the reasons listed above, EM should be performed when the flushing or aspiration method cannot be done because of the small dimension of the cauda (Hori et al., 2015). In a comparative study, the utiliza-

tion of EM yielded higher total sperm recovery (TSR) per epididymis than the aspiration method. Other authors (Hori et al., 2005) argue that EM does not differ significantly in terms of TRS compared to the flushing method (Table 1).

- d) **Aspiration** is a technique that does not require isolation of the epididymis from the testicle. The cauda epididymis is fixed by hemostatic forceps and cut using a scalpel blade. The epididymal fluid is aspirated with an automatic pipette once it begins flowing out of the incision. The aspirated material is then placed in the selected medium (Simons et al., 2019).

- Mogheiseh et al. (2022) referred to this technique as **MESA (microsurgical epididymal sperm aspiration)** and used a microhematocrit glass tube to precisely aspirate fluid obtained after several small cauda epididymal incisions. Comparison of RF with the aspiration method showed a significant difference in TSR ($372.2 \pm 12.96 \times 10^6$ vs $199.28 \pm 33.12 \times 10^6$) (Mogheiseh et al., 2022).

Some authors do not use only one technique for epididymal semen collection, combining squeezing and aspiration or mincing techniques (Garcia-Macias et al., 2006; Korochkina et al., 2014; Simons et al., 2019).

In our opinion, it is best to use squeezing and RF methods first. It is essential to remember to repeatedly and thoroughly cleanse the entire tissue of the epididymal tail from blood. In the case of obtaining semen with low concentration, other methods can be used. We recommend to start from aspiration method. When the epididymis is small the EM can be performed, but it will be associated with contamination with epithelial and blood cells.

Techniques used for the collection of sperm from in vivo epididymis

Percutaneous epididymal sperm aspiration (PESA) is a medical procedure generally performed in human ARTs (Mogheiseh et al., 2022; Simons et al., 2019; Varesi et al., 2013). In dogs, PESA can be carried out under general anesthesia when castration is not possible because of the owner’s personal reasons or behavioral reasons relating to the dog. A 26G (Varesi et al., 2013) or 24G (Mogheiseh et al., 2022) needle connected to a syringe containing the medium is inserted into the scrotal skin and guided by palpation into the epididymis cauda. It is important to maintain a negative pressure while the needle is gently moving partially in and out of the skin when changing the aspiration direction by 360° (Ali Hassan et al., 2023; Varesi et al., 2013). The limitations of the method are as follows: blood contamination may occur during collection and use of the method results in low TRS per epididymis: 2.3 to 177.5×10^6 (Varesi et al., 2013) or 29.65 ± 7.73 (Mogheiseh et al., 2022).

Epididymal semen quality assessment

The quality of semen obtained from the epididymis depends on different factors such as the time and technique of recovery.

Table 1. Characteristics of media and techniques used to collect epididymal sperm

Semen collection technique	Type of medium	Total sperm recovery outcome per epididymis	Tissue (T+) or Erythrocyte (E+) contamination	References
MINCING	Gent® semen extender (Minitüb, Tiefenbach, Germany)	742×10^6	T+	Kline et al. (2005)
	HF-10: Ham's F-10 supplemented with: glutamine, Na-benzyl penicillin, streptomycin sulfate, 5% fetal bovine serum	$104.2 (31.2 \text{ to } 620.4) \times 10^6$	T+ E+	Varesi et al. (2013)
	Canine prostatic fluid	$494.6 \pm 67.7 \times 10^6$	T+	Hori et al. (2015)
	TFC: Tris-fructose citrate solution	NO INFORMATION	T+	Fayez et al. (2023)
	DPBS	1230×10^6	E+	Ali Hassan et al. (2023)
SQUEEZING	Ringer solution without lactate	NO INFORMATION	NO	Martins et al. (2009, 2012)
ASPIRATION	0.9% saline solution	NO INFORMATION	NO	Martins et al. (2009)
	HF-10	$93 (28.4 \text{ to } 218) \times 10^6$	NO	Varesi et al. (2013)
	TALP: Tyrode's albumin lactate pyruvate supplemented with: HEPES (hydroxyethyl piperazineethanesulfonic acid), BSA: bovine serum albumin	$694 \pm 244.5 \times 10^6$	NO	Simons (2018)
	Canine prostatic fluid	$595.8 \pm 90.2 \times 10^6$	NO	Hori et al. (2015)
RETROGRADE FLUSHING	TEG: Tris-egg yolk-glucose	$551.7 \pm 59.2 \times 10^6$	NO	Batista et al. (2016)

The canine epididymal sperm collection technique allows for the determination of concentration, total sperm count, or even morphological abnormalities (Ali Hassan et al., 2023; Hori et al., 2006; Martins et al., 2009).

Epididymal sperm motility

Motility and kinetic parameters are assessed subjectively (Hori et al., 2015, 2005, 2004, 2011, 2006; Ponglowhapan and Chatdarong, 2008; Ponglowhapan et al., 2006) or through the use of a computer-assisted analysis system (CASA) (Martins et al., 2012, 2009; Mogheiseh et al., 2022).

Epididymal sperm morphology

The assessment of the morphology of epididymal sperm requires a different form of assessment due to the physiological occurrence of cytoplasmic droplets (Ali Hassan et al., 2021), which are persistent germinative residues of cytoplasm. The droplet contains enzymes and energy substrates used to support the energy of mitochondria. When performing morphological analyses, the presence of 10.3% proximal and 6.6% distal droplets should be considered as an amount that does not negatively affect mitochondrial activity and motility parameters (Angriman et al., 2017). Therefore, sperm with proximal or distal cytoplasmic droplets should not be defined as abnormal but as immature instead (Hori et al., 2015).

Changes in the morphometry and shape of the head between each part of the epididymis (the caput, corpus,

and cauda) are related to chromatin condensation. Morphometric measurement of the sperm head may be an indicator of the fertilization capacity of sperm collected from the epididymal cauda. However, it has been proven that the choice of staining technique affects the results of head and tail measurements. SpermBlue® and DiffQuick methods afford greater ellipticity and elongation; however, eosin/nigrosin staining provides the smallest sperm dimension (Wysokińska et al., 2021).

Epididymal sperm acrosome integrity

Acrosome integrity can be analyzed using fluorescent staining. In a number of studies, peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) have been used to evaluate the fluorescence intensity of the acrosomal cap (Galarza et al., 2021; Ponglowhapan et al., 2006; Varesi et al., 2013). In other studies, the chlortetracycline (CTC) assay was applied for the assessment of uniform bright fluorescence over the entire sperm head in un-capacitated acrosome intact sperm (Hewitt et al., 2001; Mogheiseh et al., 2022).

Epididymal sperm membrane integrity

The most commonly used method for the plasma membrane integrity assessment of the epididymal sperm is hypo-osmotic swelling test (HOST). In a number of studies, sperm cells were evaluated under a bright microscope and the curling of the tail was noted as an indicator of a functional plasma membrane (Fayez et al., 2023 a, b; Mogheiseh et al., 2022; Mota Filho et al., 2014). Other

authors have used fluorescent staining with carboxyfluorescein diacetate (C-FDA) and PI. In these studies, C-FDA-positive and PI-negative sperm were classified as groups with intact plasma membranes (Ponglowhapan and Chatdarong, 2008; Ponglowhapan et al., 2006). Examinations using flow cytometry and dual fluorescent staining method with PI and SYBR allow for a more accurate analysis of the cell membrane integrity status. This method shows three subpopulations: dual fluorescent cells (moribund-SYBR+ PI+), green fluorescent cells (live-SYBR+), red fluorescent cells (dead-PI+). The SYBR 14/PI staining method is successfully used in current work on canine ejaculated semen (Niżański et al., 2012; Watts, 2021), but yet we have found only one publication that included this analysis in research on canine epididymal semen (Korochkina et al., 2014).

Epididymal sperm DNA integrity

Only a few authors in the literature have assessed the DNA structure of fresh epididymal sperm. Assessment of DNA integrity and maturity appears to be of decisive importance in the assessment of epididymal sperm fertility due to the process of chromatin condensation and possible defects that occur during epididymal transit. The results of previous studies involving the use of the Sperm Chromatin Structure Assay (SCSA) confirm the fact that there are no significant changes in DNA fragmentation index (DFI) between caudal or ejaculated semen. However, there are significant differences in DFI between samples from caput and cauda of epididymis (Garcia-Macias et al., 2006).

Fertilizing ability assessment

Zona pellucida binding assay (ZBA) can be performed to determine the ability of fresh epididymal semen to penetrate oocytes. In a number of studies, before incubation, spermatozoa were diluted with TALP (Martins et al., 2009) or canine capacitation medium (CCM) and subsequently placed in droplets under oil in 5% CO₂ at 39°C for 1 h (Hewitt et al., 2001) or at 38°C for 18 h maximum (Martins et al., 2009). The percentage of spermatozoa that bound to zona pellucida or penetrated oocytes was counted following Hoechst 33258 fluorescent dye (Hewitt et al., 2001; Martins et al., 2009).

Short-term canine epididymal semen storage methods

The storage of the collected epididymis at a temperature of 20°C without altering semen quality parameters is possible for a maximum of 6 hours. According to some authors, the collected semen from the dog's epididymis can also be stored in a physiological saline solution at a temperature of 4–5°C for a maximum of 48 hours (Tittarelli et al., 2006).

Cool storage of dog epididymal semen is of lesser importance in veterinary practice, in contrast to ejaculated semen. The chilling process is used for ensuring the survival of valuable gametes during long transport to the laboratory or sperm banking center (Batista et al., 2016;

Luvoni and Morselli, 2017; Ponglowhapan et al., 2006; Rota et al., 1995; Tittarelli et al., 2006).

It is necessary to use an appropriate diluent based on an egg yolk: most often Tris-citric-fructose (TEF) (Ponglowhapan et al., 2006; Tittarelli et al., 2006) or Tris-citric-glucose (TEG) (Batista et al., 2016; Ponglowhapan et al., 2004). Extenders containing sugars that are substrates of glycolysis and oxidative phosphorylation reactions in sperm cell. These metabolic processes result in the production of energy, which is necessary during the storage period. However, researches have shown that sperm motility significantly decreases after 48 hours of preservation at 4–5°C (Batista et al., 2016; Ponglowhapan et al., 2006; Tittarelli et al., 2006). Surprisingly, thawed sperm motility is similar in samples frozen immediately after collection and after 4 days of chilling (Ponglowhapan et al., 2006).

Cryopreservation of canine epididymal semen

Extenders

Conventional freezing extenders and protocols applied for ejaculated semen are usually used for freezing epididymal sperm. One-step (Fayez et al., 2023 a, b; Martins et al., 2012, 2009; Peña and Linde-Forsberg, 2000) or two-step (Ali Hassan et al., 2023; Garcia-Macias et al., 2006; Hewitt et al., 2001) dilution using TEF (Hori et al., 2015, 2004; Ponglowhapan et al., 2006) or TEG (Mogheiseh et al., 2022) extenders have been recently used in a number of studies with different concentrations of glycerol in the second diluent (Table 2).

In a similar vein, ultra-rapid freezing of epididymal sperm was carried out with replacement of glycerol with 500 mM sucrose. However, for ultra-rapid-frozen canine epididymal semen, the protective effect of this disaccharide does not appear to be sufficient. Total and progressive sperm motility and acrosome integrity were seriously affected compared to conventional frozen samples (Galarza et al., 2021). According to other authors, canine epididymal spermatozoa show higher cryoresistance to the vitrification process in comparison with ejaculated sperm (Cerqueira Lozano et al., 2023).

The active anionic detergent sodium dodecyl sulfate (SDS) can be added alone (Mogheiseh et al., 2022) or as one of the components in Equex STM (Mogheiseh et al., 2022) or Orvus paste (Hori et al., 2015, 2005) in egg-yolk-based extenders. The essence of the beneficial effect of SDS on cell membranes is the modification of tertiary structures of yolk lipoproteins (Hori et al., 2015, 2005). Its protective effect on cryopreservation-induced acrosome and membrane damage was previously demonstrated in ejaculated semen (Nöthling et al., 2007) and was confirmed in the evaluation of post-thaw epididymal sperm (Ponglowhapan and Chatdarong, 2008).

Comparison of commercial extenders, including Bioladyl with Equex STM paste and Andromed, showed that the former is more suitable for flushing and freezing sperm from cauda because of the significantly higher percentage of progressively motile (PM) spermatozoa after

thawing (Nöthling et al., 2007). Bovimix (Martins et al., 2012) and APC-106c (Mota Filho et al., 2014) have also been used as extenders for epididymal sperm conservation.

A number of studies have been performed involving the use of diluents with egg yolk alternatives such as soybean lecithin, skim milk, polyvinyl alcohol, and low-density lipoproteins (LDL) in the process of freezing ejaculated semen (Ali Hassan et al., 2021). To the best of our knowledge, the results of the use of soybean extenders for the cryopreservation of canine epididymal semen have not been published. Prapaiwan et al. (2016) proved in their study that a 4% LDL supplementation extender has the same or better protective effect on sperm motility and membrane integrity as an egg-yolk extender.

Some authors suggest adding prostate fluid after thawing to promote a positive effect on the female reproductive tract, resulting in higher fertility (Nöthling et al., 2007). According to Hori et al. (2005), sensitizing fresh collected epididymal semen using prostatic fluid enhances sperm motility and viability after thawing.

Research into the cryopreservation of canine ejaculated semen has been recently focused on the addition of various antioxidants, including astaxanthin, resveratrol, quercetin, myoinositol, curcumin, iodixanol, spermine, kinetin, melatonin, metformin, olive-derived antioxidants, co-enzyme Q10, or even mesenchymal stem cells and their derivatives (Divar et al., 2022; Kobayashi et al., 2021; Mahiddine and Kim, 2021). This direction of

study is justified in the case of epididymal sperm due to their greater susceptibility to oxidative stress. The tendency to disrupt the integrity of cell membranes due to lipid peroxidation may result from the retention of cytoplasmic droplets or increased ROS production due to contamination with red blood cells (Ali Hassan et al., 2021). The addition of 100 µg zinc oxide nanoparticles to the TEF extender resulted in significantly higher post-thaw motility, viability, and acrosome and plasma membrane integrity (Fayez et al., 2023 a). The same parameters can be critically enhanced via 0.0035 mol melatonin supplementation of the TEF extender (Fayez et al., 2023 b).

Freezing protocols

For long-term storage of freezing epididymal sperm, 0.5 ml straws (Martins et al., 2012, 2009; Mogheiseh et al., 2022), 0.25 ml straws (Fayez et al., 2023 a, b; Galarza et al., 2021), or pellets (Galarza et al., 2021) can be used. The percentage of post-thawed sperm PM was 21.0–37.35% in 0.25 straws (Fayez et al., 2023 a, b), 4.3–40.5% in 0.5 straws (Martins et al., 2012; Mogheiseh et al., 2022), and 13.0–21.5% in pellets (Galarza et al., 2021). Horizontal sensitization in liquid nitrogen vapors in conventional styrene foam boxes (Ali Hassan et al., 2023; Hori et al., 2005, 2004, 2011; Yu et al., 2002) or three-step lowering in a tank (Korochkina et al., 2014) or programmable freezer (Varesi et al., 2014) were used as the straw freezing protocols.

Table 2. Freezing protocols, extender supplementation and assessment of their efficiency in post-thawed sperm quality parameters

Freezing protocol	Supplementation of freezing extenders	Concentration of glycerol	Post-thawed sperm quality assessment parameters	Efficiency of cryopreservation	References
TWO-STEP DILUTION	1% Equex STM paste (Extender II)	3% (Extender I) 7% (Extender II)	Sperm motility; membrane and acrosomal integrity	Improvement of assessed parameters	Ponglowhapan and Chatdarong (2008)
	4% low-density lipoprotein LDL	3% (Extender I) 7% (Extender II)	Sperm motility; membrane and acrosomal integrity; sperm head morphology		Prapaiwan et al. (2016)
	0,25% sodium dodecyl sulfate SDS (Extender II)	10% (Extender II)	Sperm motility; membrane, acrosomal and DNA integrity; morphology		Mogheiseh et al. (2022)
	500 mM sucrose (Extender II), during ultra-rapid freezing	-	Sperm motility; membrane and acrosomal integrity; sperm head morphometry	Decreased motility	Galarza et al. (2021)
	2 ml of erythrocyte lysis buffer (ELB) 1% Equex STM paste (Extender II)	3% (Extender I) 7% (Extender II)	Sperm motility; membrane integrity; morphology, intracellular reactive oxygen species (ROS), and lipid peroxidation (LPO)		Ali Hassan et al. (2023)
ONE-STEP DILUTION	–	8%	Sperm motility; membrane integrity; acrosome-reacted sperm; zona pellucida binding assay (ZBA)	Improvement of assessed parameters	Hewitt et al. (2001)
		7%	Sperm motility; membrane, acrosomal and DNA integrity; LPO		Fayez et al. (2023 a)
	0.0035 mol melatonin	7%	Sperm motility; membrane, acrosomal and DNA integrity; LPO		Fayez et al. (2023 b)

Table 3. Comparison of the total motility of fresh epididymal sperm collected using a specific method and frozen–thawed sperm after cryopreservation in a particular extender

Collection method	TM (%) of fresh epididymal semen		Freezing extender	TM (%) of frozen–thawed epididymal semen		References
	Result (mean±SEM)	Assessment method		Result (mean±SEM)	Assessment method	
EM	89.4±1.6	SLM	TEF	19.5±2.5	SLM	Hori et al. (2003)
RF	67.8±9.5	SLM	TEG	35.3±13.6	SLM	Ponglowhapan et al. (2006)
SQUEEZING	80.0	CASA	Bovimix®	69.3±3.2	CASA	Martins et al. (2012)
EM	87.2±3.9	SLM	TEF	20.0±3.8	SLM	Hori et al. (2015)
RF	92.2±0.9	SLM	TEF	26.7±2.7	SLM	Hori et al. (2015)
RF + EM	75.5±3.63	CASA	TEG	37.7±4.43	CASA	Galarza et al. (2021)
MESA	92.19±3.69	CASA	TEG	58.00±10.08	CASA	Mogheiseh et al. (2022)
RF	85.50±7.88	CASA	TEF	45.31±7.72	CASA	Mogheiseh et al. (2022)
EM	“greater than 70%”	SLM	TEF with 0.0035 mol melatonin	33.00±2.54	SLM	Fayez et al. (2023 a)
EM	“greater than 70%”	SLM	TEF with 100 µg/ml ZnONPs	37.00±2.54	SLM	Fayez et al. (2023 b)

EM – epididymal mincing; RF – retrograde flushing; TM – total motility; SLM – subjectively in light microscope; CASA – computer-assisted sperm analysis; TEF – Tris-egg yolk-fructose extender; TEG – Tris-egg yolk-glucose extender.

Assessment of frozen-thawed epididymal sperm

The authors of published studies mainly assess parameters of motility or integrity of the sperm membranes or acrosomes of frozen–thawed semen as determinants of freezability using the methods described in Section 3 of the present review (Fayez et al., 2023 a, b; Galarza et al., 2021; Mogheiseh et al., 2022). Mean values of total frozen semen motility can be assessed subjectively by light microscopy or by CASA (Table 3).

Considering only the motility parameter, the use of the squeezing method to obtain sperm from the epididymis and then freezing in the Biovimix® extender gives the best results. It should be noted, however, that the authors did not examine other parameters of sperm quality like acrosome and sperm integrity or DNA integrity.

Ali Hassan et al. (2023) performed an evaluation of reactive oxygen species (ROS) and lipid peroxidation (LP) in frozen–thawed sperm. The authors revealed that the use of erythrocyte lysis buffer (ELB) did not improve the mentioned parameters. ROS content was determined using nitroblue tetrazolium and values of 70% (standard freezing protocol) and 78.5% (ELB freezing protocol) were achieved. LP was assessed using thiobarbituric acid reactive substances (TBARS) measured via spectrophotometry. The values did not differ significantly between standard and ELB freezing protocols and gave respective values of 3.6 ng and 4.3 ng TBARS/10⁶ spz.

It should be noted that the use of advanced methods for assessing epididymal semen is still limited. A comparison of SCSA and the Sperm Chromatin Dispersion test (SCDt) as an assessment for the DNA fragmentation of frozen–thawed epididymal sperm revealed that both

techniques give similar findings and can be used to extensively evaluate DNA changes (Ortiz et al., 2017). The importance of DFI results may be limited due to the lack of an established index value in dogs. For fertile dogs, the mean DFI ejaculated semen value is 5.9%, and for infertile dogs, this value stands at 13.2% (Nizański et al., 2011). During their review of the available literature, the authors did not find any studies that used flow cytometric methods, including SCSA-Acridine orange (AO); comet assay; or TUNEL for epididymal sperm DNA assessment (Chatdarong et al., 2012; Fayez et al., 2023 a; Nizański et al., 2011).

Use of proteomic methods for assessment of canine epididymal semen

One method that has seen increasing popularity in animal reproduction is **proteomics**. The focus of researchers' interest is the detection and characterization of proteins in sperm membranes and intracellular structures (Gouletsou et al., 2022; Zmudzinska et al., 2022 a), seminal plasma (Aquino-Cortez et al., 2017; Araujo et al., 2022), and epididymal fluid (Cichowska et al., 2023). Proteomic analysis involves the use of polyacrylamide gel electrophoresis with nanoliquid chromatography coupled to mass spectrometry or with Western blotting (Aquino-Cortez et al., 2017; Angrimani et al., 2017; Cichowska et al., 2023; Galarza et al., 2021; Zmudzinska et al., 2022 a, b).

Proteomic studies of protein localization and function in metabolic pathways make it possible to identify markers of semen quality or freezability (Zmudzinska et al., 2022 a). In a study, the use of proteomic analy-

sis of epididymal fluid allowed for the identification of six unique proteins indicative of good sperm motility (Cichowska et al., 2023). Transfer, carrier, enzyme, and cytoskeletal proteins are the most dominant protein classes in epididymal fluid and create a unique environment for sperm regulation and maturation.

The proteomic characteristics of the epididymal spermatozoa of dogs in different age groups resulted in the identification of unique proteins for young (1–3.5 years of age), adult (3.6–6.5 years of age), and old (6.6–11 years of age) male dogs. Age-dependent changes were detected among the study groups as a result of the proteome composition and semi-quantitative analysis of certain proteins, which suggests the use of these proteins as sperm aging markers (Zmudzinska et al., 2022 b).

Practical application of preserved canine epididymal semen

The insemination of frozen–thawed semen is performed via non-surgical transcervical catheterization or surgical procedures (laparoscopy or laparotomy) for intrauterine deposition (Nizański et al., 2012).

The use of epididymal semen for insemination is particularly important for valuable stud dogs excluded from breeding because of prostatic gland dysfunction. Benign prostatic hyperplasia (BPH) is the most prevalent prostatic disease diagnosed in 80% of dogs over 6 years of age and 95% of dogs over 9 years of age (Domosławska et al., 2023; Khaki et al., 217; Ruetten et al., 2021). Research results confirm that 17% of affected males do not respond to drug therapy with osaterone acetate (Socha et al., 2018). In such cases, after the exclusion of the neoplasm process in prostatic tissue, the best form of treatment is castration (Ruetten et al., 2021). It has been proven that pregnancy and the birth of healthy litters are possible after insemination with epididymal semen collected from dog testis after castration due to BPH (Klinc et al., 2005). In a study, fertility after intrauterine insemination of frozen 200×10^6 sperm per dosage resulted in a low conception rate when using cauda epididymal (12.5%) or ejaculated sperm (25.0%) (Hori et al., 2011). The results of a number of studies confirm the fact that epididymal semen shows decreased motility after cryopreservation compared to ejaculated frozen sperm. Considering that fact, Hori et al. (2004) increased the number of epididymal sperm for intrauterine laparotomy insemination. The authors compared conception results after applying 2×10^8 , 3×10^8 , and 4×10^8 sperm per dosage. In this experiment, one female inseminated with a dose of 3×10^8 spermatozoa became pregnant and gave birth to two puppies (conception rate of 6.3%). As early as 1994, a Boxer female was surgically inseminated with sperm from an epididymis stored in pellets. Interestingly, the procedure resulted in the birth of one puppy, despite the fact that the thawed sperm's progressive motility was 5% (Marks et al., 1994). This result proves the significant importance of examining the structure of DNA, the integrity of the plasma

acrosome membrane, or mitochondria activity in assessing the fertilization capacity of cryopreserved sperm.

Conclusions

Success in achieving high-quality frozen insemination doses of dog epididymal semen depends on many factors. To conclude from the obtained data, the method for collection epididymal semen should be adapted to the size and the quality of epididymal tissue. The optimal storage medium and temperature should be selected taking into account the transport time to the cryopreservation center. In summary, the use of an egg yolk-based extender appears to be the most efficient method for cryopreservation of epididymal sperm. We would like to emphasize that the examination of epididymal semen quality should be extended to include: the mitochondrial status, the degree of DNA damage, ROS levels or proteomic characterization. All the more so, there are a limited number of publications on the practical (insemination) use of frozen epididymal sperm and its outcomes and effectiveness in terms of conception rates. Conversely, the limited scope of *in vivo* research is understandable in the case of privately owned, companion, and high-breeding-value animals.

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