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Testicular cryopreservation in dogs and cats

Criopreservação testicular em cães e gatos

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Abstract

Reproductive biotechniques play an important role in optimizing reproductive processes. Among them, testicular cryopreservation stands out as an innovative and valuable technique. Its significance lies in several key aspects, such as preservation of fertility potential; genetic conservation; assisted reproduction and its contribution to advances in Veterinary Medicine and research. Furthermore, testicular cryopreservation is essential for conservation programs involving both wild and domestic species. Dogs and cats are of particular interest in studies of reproductive biotechnology, given their increasing global relevance and their potential as experimental models for wild canines and felines. Moreover, this biotechnique offers the unique advantage of preserving the gonads of prepubertal animals or when semen collection is not feasible. Additionally, it serves as a reliable source of male gametes, maintaining spermatogenic potential. However, despite the advances already made in testicular cryopreservation, a standardized protocol suitable for clinical implementation in the testicular preservation of dogs and cats has yet to be established. Therefore, the aim of this review was to explore the aspects of testicular cryopreservation in dogs and cats with the main findings obtained to date.

Keywords*: companion animals, male gonad, conservation, vitrification, freezing.*

Introduction

Dogs and cats are the most common domesticated species in homes around the world. They have developed exceptionally strong and successful relationships with humans. In Brazil, between 2020 and 2021, the dog population increased by 3.9%, reaching 58.1 million, while the cat population grew by 5.9%, totaling 27.1 million, highlighting the growing importance of these animals in society (Abinpet, 2023). Therefore, with the increasing breeding of dogs and cats, it is natural to be interested in improving the reproductive potential of these species, whether for commercial breeding or as companion animals (Silva, 2022). In this sense, testicular cryopreservation is a prospected reproductive biotechnique, bringing another alternative for means of assisted reproduction, when the individual's semen or epididymal sperm are not an available possibility (Picazo et al., 2022).

The development of research on testicular conservation in domestic animals is supported by several reasons (Lima et al., 2018a). Among them, the use of these animals as experimental models for phylogenetically related wild species (Comizzoli and Holt, 2014; Andrae et al., 2021) and even for comparison with more distantly related species, such as wild boar, respecting their biological particularities (Picazo et al., 2022). In addition, such studies contribute to the preservation and dissemination of germplasm of zootechnical interest. Studies involving dogs and cats also play a crucial role in the development of experimental disease models, including clinical applications aimed at preserving reproductive potential in humans, especially in patients who have experienced depletion of male germ cells due to treatments such as radiotherapy and/or chemotherapy for cancer (Kim et al., 2008).

Testicular cryopreservation has been developed in different species, including cats (Lima et al., 2018a) and dogs (Carvalho, 2016; Teixeira et al., 2021). However, further research is needed in both species to refine and standardize effective cryopreservation protocols.

Therefore, the objective of this review was to survey testicular cryopreservation, the factors that influence the efficiency of testicular cryopreservation, as well as the post-thawing or heating evaluation methods in canine and feline species with the main findings obtained to date.

Testicular cryopreservation

Cryopreservation is a process that seeks to preserve cells or tissues by cooling samples to very low temperatures, allowing the conservation of cells in a viable state for long periods (Jang et al., 2017).

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Testicular cryopreservation enables the preservation of reproductive potential and allows the creation of germplasm banks for species of commercial interest or at risk of extinction (Oliveira, 2015; Lima and Silva, 2018). In addition, it enables both the transport of genetic material between different regions (Lima and Silva, 2017) as well as its storage for an indefinite period. It is important to highlight that this biotechnology represents the only alternative for preserving fertility and using the gonads of deceased prepubertal animals, being essential in recovering and disseminating genetic material that would otherwise be lost (Pukazhenthi et al., 2015).

Testicular cryopreservation enables the culture of numerous germ cells at various developmental stages, including undifferentiated spermatogonia, potentially allowing the continuous sperm production (Comizzoli and Holt, 2014; Silva et al., 2020).

Testicular cryopreservation methods

The main methods of testicular cryopreservation are slow freezing and vitrification (ultra-fast freezing) (Silva, 2022). In the cryopreservation process, cryoprotectants should be used to minimize the formation of ice crystals.

There are no studies comparing slow freezing to vitrification in cats. However, in dogs, a study evaluated these techniques in testicular samples from pubescent dogs and found that needle immersed vitrification was more effective in preserving the viability and DNA integrity of round cells (Picazo et al., 2022; Table 1).

Slow freezing

Slow freezing consists of a gradual reduction in temperature, associated with low concentrations of cryoprotectants, reducing their toxicity (Keros et al., 2005). However, its ability to prevent the formation of intra- and extracellular ice crystals is limited (Dobrinsky, 1996), which can cause cryoinjury to cells (Chang and Zhao, 2021). Furthermore, although slow freezing is effective in preserving the morphological characteristics of the seminiferous tubules, it has some specifications, such as the complexity of the process, the time required for its execution – which can last more than 24 hours (Pothana et al., 2015) – and the high cost of the equipment involved (Radaelli et al., 2017).

The process occurs as follows: 1. The fragments are incubated in a freezing medium; 2. Then they are inserted into a freezing equipment. 3. Following, the samples are stored in liquid nitrogen. 4. Finally, the samples are thawed and the cryoprotectants are removed (Schlatt et al., 2002).

Slow freezing can be performed using two different systems:

Controlled slow freezing: Uses specialized equipment (programmable freezer or specific container, such as Mr Frosty®) that gradually reduces the temperature of the samples at a specific and constant rate (automated and controlled) until they reach extremely low temperatures, which are then stored in liquid nitrogen (Pukazhenthi et al., 2015; Lierman et al., 2021). An essential aspect of this protocol is seeding (Kvist et al., 2006), a technique that induces the formation of ice crystals in a controlled manner during the freezing process (Tan et al., 2021). Among the benefits of this method, high reproducibility and precise control offered by the equipment stand out, which are fundamental factors for the success of cryopreservation.

Uncontrolled slow freezing: Does not use programmable equipment, depending on manual variations or less precise methods for temperature reduction (Gomes, 2024).

Vitrification

Vitrification is a promising cryopreservation method for testicular samples, offering advantages such as lower cost and faster execution compared to slow freezing (Fernandes et al., 2021). This method, widely used since the 1980s, consists of ultra-fast freezing (rapid reduction in temperature) and the use of solutions with high concentrations of cryoprotectants. As the temperature decreases, the solutions increase in viscosity leading to the formation of an amorphous solid or pseudo-vitreous state (Fuller and Paynter,

Table 1: Overview of studies on testicular cryopreservation in dogs.

Goals	Technical approach	Main results	Reference
To compare different cryoprotectants. To compare SF and SSV.	SF with 10% DMSO or 7% GLY and SSV with 15% DMSO/200mM trehalose or 7% GLY/200mM trehalose.	SSV with DMSO and trehalose produced better integrity of structure and architecture of the samples.	Carvalho, 2016
To identify the ideal conditions for freezing canine testicular cells for GDC culture. Determine the spermatogonial stem cell capacity of these GDCs.	SF with 10% DMSO and *in vivo* or *in vitro* culture.	DMSO as a cryoprotectant in freezing and StemPro 34 SFM as a culture medium for canine testicular cells were suitable, providing viable cells to develop during *in vivo* and *in vitro* culture.	Lee et al., 2016
To compare different CRYO. To compare SF and SSV.	SF and SSV with DMSO and EG isolated ([30%]) or combined ([15%] each).	DMSO/EG in SF achieved better results.	Santos, 2018
To compare two immersion times in freezing medium.	SF in 15 or 30 minutes of immersion.	The immersion times and the freezing technique used were efficient.	Silva et al., 2017
To histologically evaluate the testes of prepubertal dogs with different cryoprotectants associations using SSV.	SSV with DMSO/GLY, DMSO/EG or GLY/EG (2,8 M of each cryoprotectant).	EG/GLY and DMSO/EG were the ones that best preserved testicular integrity.	Teixeira et al., 2021
To compare the integrity of germ cells in dogs after SF or NIV.	SF with DMSO 2,8 M and NIV with 2,1M DMSO/2,7M EG.	NIV better preserved the viability and DNA integrity of rounded cells.	Picazo et al., 2022
To compare different cryoprotectants associations.	SSV using DMSO/EG, DMSO/GLY or EG/GLY (2,8 M of each cryoprotectant).	DMSO/EG was the most effective in preserving testicular histomorphological structure.	Noronha, 2024

DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; GDC: Germ cell-derived colonies; GLY: Glycerol; NIV: Needle immersed vitrification; SF: Slow freezing; SSV: Solid surface vitrification.

Table 2: Overview of studies on testicular cryopreservation in cats.

Goals	Technical approach	Main results	Reference
Focus on testicular collection, cryopreservation and storage in a cooled environment of testicular fragments, to optimize conditions for later application in endangered felines.	Refrigeration at 4 °C or SF with DMSO.	Testicular cryopreservation with DMSO failed to produce germ cell grafts. In contrast, testes from prepubertal animals could be preserved in cold medium for 2 to 5 days, while pubertal testes have demonstrated to be more susceptible to hypoxia/storage in culture medium. Testicular weight can be used to predict the success of xenotransplantation and help predict the number of mice to be used.	Mota et al., 2012
To determine the effects of cryoprotectants and freezing protocols on the plasma membrane of testicular spermatozoa, DNA integrity and fertilization capacity after ICSI.	Two-step freezing vs. controlled SF using GLY, EG, PRO or DMSO.	The testes were successfully cryopreserved. The types of cryoprotectants and freezing techniques play a central role in determining the post-thaw quality of feline testicular sperm. Frozen testicular sperm maintain fertilization capacity, although the developmental capacity of embryos derived from ICSI with frozen testicular sperm is low.	Buarpung et al., 2013
To evaluate testicular cryopreservation by comparing two fragment sizes (0.3 and 0.5 cm^3) and two cryoprotectants (GLY 3% and PRO 3%).	SF	GLY was more efficient than PRO in SF. Fragments of 0.5 cm^3 showed better results.	Macente et al., 2017
To evaluate the effect of different cryoprotectants associations on testicular integrity and spermatogonial proliferation potential after testicular vitrification in prepubertal cats.	SSV with DMSO/EG, DMSO/GLY or EG/GLY (2.8M of each cryoprotectant)	DMSO/GLY showed the best testicular preservation and the greatest potential for cell proliferation after vitrification.	Lima et al., 2017

To study the structural and functional properties of testicular fragments from prepubertal cats after vitrification followed by two warming protocols (directly to 37°C or with a 5-s pre-exposure to 50°C) and three resuscitation time points (immediately, 24 hours, and 5 days after warming).	NIV	The preservation of seminiferous tubule structure was better using warming at 50°C for 5 seconds, and survival of somatic and germ cells was higher compared with direct warming at 37°C for 1 minute. Short-term *in vitro* culture also demonstrated that cellular composition and functionality were better preserved when warming was performed for a short period at 50°C. Brief warming at 50°C led to improved quality of seminiferous tubule structure and cellular composition after vitrification and short-term culture.	Lima et al., 2018a
To evaluate the effect of different cryoprotectants associations on testicular integrity, potential for cell proliferation and viability of germ cells after storage in cryotubes.	Vitrification in cryotubes	Vitrification in cryotubes can be successfully used for testicular cryopreservation. DMSO/GLY contributed most to the maintenance of testicular histomorphometric characteristics after vitrification.	Lima et al., 2018b
To evaluate DNA damage and estimate apoptosis rates in testicular fragments. The values for these variables were compared between the type of cryoprotectant used (3% GLY and 3% PRO) and the size of the testicular fragment (0.3 and 0.5 cm^3).	SF	Both GLY and PRO, at 3% concentration, provided protection against damage caused by cryopreservation for both fragment sizes. However, there were differences in the efficacy of cryoprotectants in terms of protective capacity, depending on the cell type.	Macente et al., 2019
To evaluate the influence of different heating temperatures (50, 55 and 60°C) on the structure, metabolic activity, composition and cellular functions of	NIV	Vitrified testicular fragments from prepubertal cats have better preservation of morphology, morphometry and viability when heated to 50 °C.	Fernandes et al., 2021

vitrified testicular fragments from prepubertal cats.			
To evaluate testicular integrity after vitrification with different devices, followed by different warming conditions.	Cryotop® and NIV/ Warming directly to 37 °C or with a 10 s pre-exposure at 50 °C	Vitrification with Cryotop®, followed by warming at 37 °C and culture at 38.5 °C for 24 hours, efficiently preserved testicular fragments and cell quality.	Macente et al., 2022
Compare different vitrification methods (OTC, straws and SSV).	OTC, straws and SSV using 40% EG + 0.1M sucrose	Although all tested devices showed morphological preservation, high cell viability and reduced apoptotic index, SSV and OTC were the most efficient in maintaining testicular integrity/viability.	Carvalho et al., 2023
To compare the effect of the isolated use and association of penetrating and non-penetrating cryoprotectants in SSV.	SSV with EG (40%) alone, associated with DMSO (20% EG + 20% DMSO), combined or not with sucrose (0.1 M or 0.5 M) or trehalose (0.1 M or 0.5 M)	EG with 0.1 M sucrose or trehalose are the most suitable cryoprotectants to preserve the testicular histological structure of adult cats.	Carvalho et al., 2024

DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; GLY: Glycerol; ICSI: Intracytoplasmic sperm injection; OTC: Ovarian tissue cryosystem; PRO: Propanediol; NIV: Needle immersed vitrification; SSV: Solid surface vitrification.

2004; Picazo et al., 2022). This process helps to better preserve cell morphology and viability (Fahy et al., 1987; Portillo et al., 2006; Poels et al., 2012; Lima et al., 2018b).

Although testicular cryopreservation by vitrification is a relatively recent technique, it has already demonstrated promising results, such as the development of flagellated sperm and functional Leydig cells in testicular fragments in samples from mice after organotypic culture (Baert et al., 2012; Dumont et al., 2015; Yildiz et al., 2018; Oblette et al., 2019), as well as prepubertal Rhesus monkeys (Poels et al., 2012) and the birth of offspring from pigs (Kaneko et al., 2013), mice (Yokonishi et al., 2014) and Rhesus monkey (Fayomi et al., 2019). However, despite the advances already achieved in other species, such progress has not yet been accomplished in dogs and cats (Silva, 2022).

Two techniques used for testicular vitrification stand out: solid surface and needle immersed vitrification. Solid surface vitrification is a simple and economical method (Da Silva et al., 2019) that consists of exposing testicular fragments to a cryoprotectant solution. Then, they are placed directly on a metal surface (Fig. 1) pre-cooled to -180 °C by partial immersion in liquid nitrogen. The fragments are then placed in cryovials and stored in liquid nitrogen tanks (Xing et al., 2010; Lima et al., 2017).

In dogs, solid surface vitrification has been proven effective in preserving the testicular histomorphological structure (Teixeira et al., 2021; Noronha, 2024). In cats, a study was carried out to compare different methods (ovarian tissue cryosystem, straws and solid surface vitrification) for vitrification of feline testicular fragments. Solid surface vitrification stood out among the methods evaluated, demonstrating better morphological preservation, high cell viability and greater efficiency in maintaining testicular integrity (Carvalho et al., 2023; Table 2).

Needle immersed vitrification (Fig. 2) was developed allowing vitrification with lower concentrations of cryoprotectants (Wang et al., 2008). This method consists of transfixing several testicular fragments in a needle (acupuncture or hypodermic needle). Thus, all samples can be exposed to cryoprotectants and immersed in liquid nitrogen under the same conditions, maximizing the cooling rate and simplifying the vitrification process (Wang et al., 2008; Fernandes et al., 2021). Although this technique presents a higher risk of contamination, a study with testicular fragments from dogs indicated that needle immersed vitrification was more efficient as the solid surface vitrification method in preserving the morphology of the seminiferous tubules (Fernandes et al., 2024).

The device called Cryotop® was used to vitrify feline testicular fragments. Results showed that testicular fragments were efficiently cryopreserved (maintaining the quality of all cell types) with vitrification with Cryotop® followed by direct warming at 37 °C, and additional culture of 24 hours at 38.5 °C. These findings demonstrate that Cryotop® is a viable option for feline testicular vitrification (Macente, et al., 2022; Table 2).

Factors influencing the effectiveness of testicular cryopreservation

Among the factors that can influence the effectiveness of testicular cryopreservation, cryoprotectants, the age of the animal, the size of the testicular fragment and the thawing or heating temperature can be mentioned.

Cryoprotectants

Cryoprotectants are chemical substances used to protect cells and tissues from damage caused by cryopreservation. Their addition is a crucial step in maintaining cell viability after thawing/warming, since the cryopreservation process can compromise the function of spermatogenic cells due to the exposure of organelles to low temperatures and osmotic imbalance (Fahy et al., 1987). Cryoprotectants act by reducing the amount of free water inside and outside the cells, preventing structural damage during freezing and thawing. They also help avoiding osmotic shock by balancing the water flow into and out the cell. They help prevent the formation of ice crystals, which can rupture cell membranes and compromise cell viability, since spermatogenic cells must remain viable for future use in assisted reproduction programs (Buarpung et al., 2013).

Cryoprotectants have been widely and successfully used in the preservation of various biological systems. Understanding the physical, chemical, and molecular characteristics of cryoprotectants is essential to predict and control their effects on biological samples, as well as to select the cryoprotectants that can promote better results (Fahy et al., 1987). Cell toxicity and osmotic shock resulting from the use of high concentrations of cryoprotectants, or prolonged exposure time represent significant challenges in the

formulation of effective cryopreservation protocols (Fahy et al., 1987; Fuller and Paynter, 2004; Yokonshi and Ogawa, 2016).

Figure 1: Metal plate for solid surface vitrification.

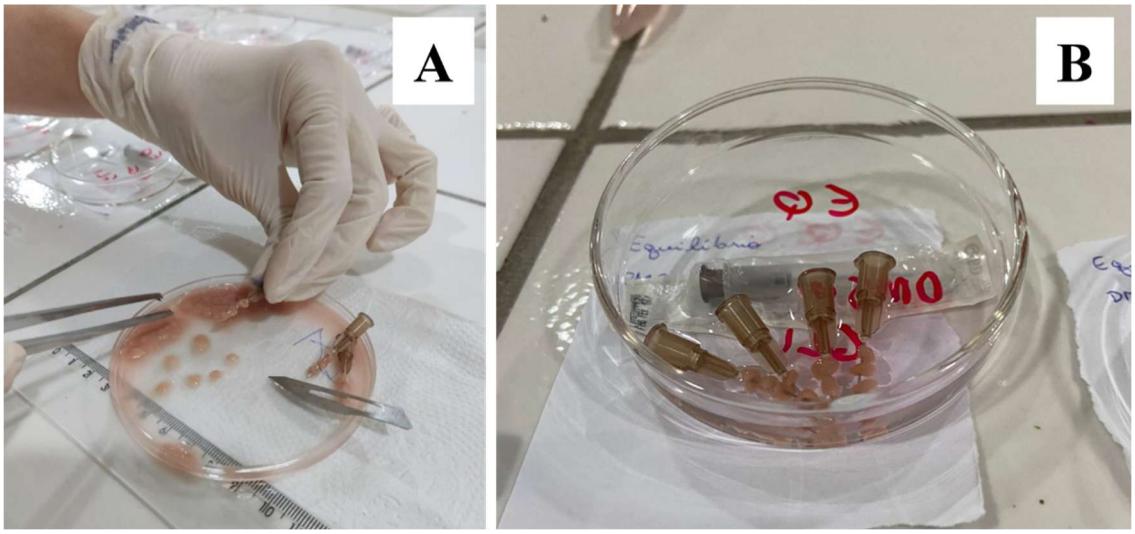

Figure 2: A. Canine testicular fragmentation for needle placement. B. Fragments already transfixed for subsequent exposure to equilibrium solution for needle immersed vitrification.

Cryoprotectants are classified as penetrating (e.g., dimethyl sulfoxide, glycerol, ethylene glycol and propylene glycol) and non-penetrating (e.g., sucrose, trehalose, serum albumin and polyvinylpyrrolidone). Intra and extracellular cryoprotectants are used to minimize the deleterious effects of cryopreservation (Chazotte, 2011; Lima et al., 2018b) and allow spermatogenic cells to remain viable

for future use in assisted reproduction programs (Buarpung et al., 2013). However, these agents, when added in high concentrations, can also cause cell damage.

Extracellular or non-penetrating cryoprotectants are high molecular weight substances that increase the osmolarity of the external environment, promoting rapid cell dehydration and thereby preventing intracellular ice crystal formation (Caturla-Sánchez et al., 2018; Chang and Zhao, 2021). The extracellular cryoprotectant most frequently used in testicular cryopreservation research is sucrose (Baert et al., 2012), followed by trehalose (Carvalho, 2016). Both sugars can be present in protocols associated with intracellular cryoprotectants (Baert et al., 2012).

On the other hand, intracellular or penetrating cryoprotectants, which have low molecular weight, act by partially replacing intracellular water and binding to its hydrogen atoms (Jain and Paulson, 2006). This increases the viscosity of the freezing solution, consequently reducing the freezing point. Additionally, these cryoprotectants help prevent exposure of the material to high electrolytes concentrations by either binding to the electrolytes or replacing water partially (Castro et al., 2011).

Dimethyl sulfoxide, ethylene glycol, and glycerol stand out among the penetrating cryoprotectants used in testicular cryopreservation protocols (Lima et al., 2018a). Dimethyl sulfoxide has demonstrated satisfactory results in preserving the integrity of samples in mice (Gossens et al., 2008). It interacts with the lipid membrane and induces the formation of pores for water flow, causing cellular dehydration and thereby minimizing intracellular ice crystal formation during cryopreservation (Gurtovenko and Anwar, 2007). On the other hand, ethylene glycol is able to permeate the cell membrane more quickly (Cooper et al., 2008), resulting in lower osmotic stress and preventing the formation of ice crystals during cryopreservation (Weng et al., 2011). Glycerol is a highly permeable polyhydric alcohol that binds hydrogen ions in the water molecule and slows down osmotic dehydration, minimizing cellular damage. In addition, it lowers the freezing point, decreases electrolyte concentrations in the unfrozen fraction of the sample, and minimizes intracellular ice crystal formation (Doebbler, 1966; Watson, 2000).

The cryoprotectants glycerol 3% and propanediol 3% were tested in the freezing of feline testes fragments associated with Tris-Equex egg yolk. The fresh and cryopreserved material exhibited similar morphology regarding the detachment of the basement membrane and the visualization of the nucleus. The main alteration identified was pyknosis, observed in approximately 50% of the fragments cryopreserved with propanediol and 53% of those preserved in glycerol. In general, the fragments frozen in propanediol presented more pronounced lesions, indicating that propanediol demonstrated greater toxicity compared to glycerol at the concentration used in this study (Sousa et al., 2017).

Feline testes were submitted to slow freezing to evaluate DNA damage and estimate apoptosis rates in testicular fragments using glycerol and propanediol and observed that both cryoprotectants at 3% concentration, provided protection against damage caused by cryopreservation (Macente et al., 2019; Table 2).

The associations ethylene glycol/sucrose 0.1 M and ethylene glycol/trehalose 0.1 M were tested in the feline testicular vitrification. The association with sucrose showed integrity in 88% of spermatogonia, 70% spermatocytes, 89% spermatids and 69% Sertoli cells; while that with trehalose showed 88% of spermatogonia, 75% spermatocytes, 90% spermatids and 60% Sertoli cells. Both treatments were inferior to the control, however they did not differ from each other. The basement membrane of the seminiferous tubules showed moderate detachment with both sucrose and trehalose. This study showed that the association of both extracellular cryoprotectants in the vitrification solution were equally efficient for cat testicular vitrification, presenting good morphological preservation of germ cells and integrity of the seminiferous tubules (Carvalho et al. 2017).

The cryoprotectants dimethyl sulfoxide, glycerol and ethylene glycol were tested separately with trehalose comparing slow freezing and vitrification for testicular cryopreservation in adult dogs. Better morphological and ultrastructural maintenance was obtained with vitrification using dimethyl sulfoxide and trehalose (Carvalho, 2016; Table 1). The cryoprotectants dimethyl sulfoxide, glycerol and ethylene glycol were also tested in association, using solid surface vitrification and it was found that dimethyl sulfoxide/ ethylene glycol was the most effective in preserving testicular histomorphological structure in the dog (Noronha, 2024; Table 1).

StemPro-34 SFM, used as an *in vitro* culture medium, and dimethyl sulfoxide, as a cryoprotectant, were shown to be effective for freezing canine testicular cells, supporting the development of viable colony-derived germ cells during *in vitro* culture (Lee et al., 2016; Table 1). The best preservation of sperm membrane and chromatin integrity in dogs after slow freezing of the testis was achieved using the association of dimethyl sulfoxide with ethylene glycol (Santos, 2018; Table 1). Findings from the vitrification of testes from dogs (Teixeira et al., 2021; Table 1) and prepubertal gray wolves (*Canis lupus*)

(Andrae et al., 2021), using the solid surface method, further support that the association of dimethyl sulfoxide/ethylene glycol better preserved testicular integrity, as assessed histologically. Good morphological maintenance and spermatogonial and Sertoli cell viability in testicular vitrification with the association of dimethyl sulfoxide/ethylene glycol of pubescent canine and pubescent wild boar testicles were achieved (Picazo et al., 2022; Table 1).

In the study in which three different cryoprotectant associations were evaluated in pairs (dimethyl sulfoxide/glycerol, ethylene glycol/glycerol and dimethyl sulfoxide/ethylene glycol) for testicular vitrification of prepubertal cats, greater separation of the basement membrane, greater degree of basement membrane retraction, worse cell distinction, worse nuclear visualization and condensation were observed in the dimethyl sulfoxide/ethylene glycol group, while the dimethyl sulfoxide/glycerol association was the one that presented the best results in these same parameters (Lima et al., 2017; Table 2).

Age (prepubertal vs. pubertal)

The age of the animal at the time of cryopreservation process can have an important influence on post-vitrification results. The goal of testicular cryopreservation is to achieve resumption of spermatogenesis in order to obtain viable spermatozoa, capable of fertilizing an oocyte. In a study that aimed to verify how the age of the donor of testicular fragments affects the progression of spermatogenesis after xenografting, it was observed that immature and young dogs (< 6 months of age) were the most promising donors (Abrishami et al., 2010). Although this study was not performed with cryopreserved material, this strategy may offer an alternative for preserving the male germline for canines that die prematurely or must be neutered before maturation.

In prepubertal animals, the seminiferous tubules are predominantly composed of spermatogonial stem cells and immature Sertoli cells. Thus, due to the presence of undifferentiated cells, the seminiferous tubules of these animals present low metabolic activity and are therefore more resistant to cryopreservation (Fayomi et al., 2019). Corroborating this, testicular fragments from prepubertal and pubertal domestic cats were cryopreserved with dimethyl sulfoxide. Xenografts were then performed in immunosuppressed mice, and it was observed that after 10 weeks of xenografting, the fragments from prepubertal animals presented a greater quantity of stem cells and seminiferous tubules in relation to pubertal animals (Mota et al., 2012; Table 2).

After vitrification of testicular cells from prepubertal dogs followed by *in vitro* culture and xenotransplantation into the subcutaneous tissue of mice, the formation of spermatogonial stem cells and seminiferous tubules was observed (Lee et al., 2016; Table 1).

Testicular fragment size

Hypothetically, there would be three possible presentations for testicular cryopreservation: cell suspension, small fragments or whole gonad. Cell suspension has some disadvantages, including a labor-intensive preparation process and negative effects on cell proliferation and differentiation, since there is no cell-to-cell interaction once they are no longer in the microenvironment of the testicular parenchyma. The preservation of the whole gonad, although a probable form, has not been reported. Therefore, most research has worked with small testicular fragments (Milazzo et al., 2008).

To offer cryoprotection, cryoprotectants need to diffuse rapidly into and out of the tissue. Therefore, the size of the testicular samples submitted to cryopreservation may be an important consideration (Honaramooz, 2012). Slow freezing of different sizes of cat testicular fragments was performed and the authors observed an increase in pyknosis in the smaller samples (Macente et al., 2017; Table 2). In dog testicular cryopreservation studies, fragment sizes from 3 to 5 mm^3 (Carvalho, 2016; Table 1) and 4 mm^3 (Silva et al., 2017; Santos, 2018) were used, however, no comparisons were made among sizes.

Thawing or warming temperature

Warming and cryoprotectant removal procedures are also critical factors for cell survival (Bagchi et al., 2008). The literature states that when freezing is slow, thawing should also be slow, and in rapid freezing, thawing should follow the same rhythm (Whittingham et al., 1972). To prevent osmotic stress and potential cell damage from excessive hydration during the warming process, it is recommended that cells be gradually exposed to decreasing concentrations of cryoprotectants (Pegg, 2007).

Post-vitrification warming occurs in a water bath at 37 ºC for 30 seconds to 1 minute or at 50 ºC for 5 seconds and then the samples are incubated in solutions of decreasing concentrations of extracellular cryoprotectants, such as sucrose (Carvalho, 2016; Fernandes et al., 2021).

In prepubertal cats, temperatures of 50, 55 and 60 ºC were tested for warming vitrified samples and the temperature of 50 ºC showed the best results (Fernandes et al., 2021; Table 2).

Post-thawing or warming evaluation methods

For cryopreservation reproductive biotechnology to be considered successful, the cells must remain viable and functional after being subjected to thermal stress (Lima and Silva, 2017). To achieve this, the effects caused by the cryopreservation process on testicular fragments must be evaluated using different predictive techniques to ensure the preservation of the integrity and viability of the material.

Histomorphometric analysis

Historphometric analysis allows to identify whether there was a change in the diameter of the seminiferous tubules. Studies evaluating tubular diameter following testicular vitrification have reported a significant reduction in this parameter (Fig. 3), in both cats (Fernandes et al., 2021) and dogs (Noronha, 2024). A possible explanation for this may be the thermal stress to which the seminiferous tubules are subjected during vitrification. However, other studies suggest that tubular diameter may recover after *in vitro* culture or xenografting of the fragments (Wyns et al., 2007; Ma et al., 2022).

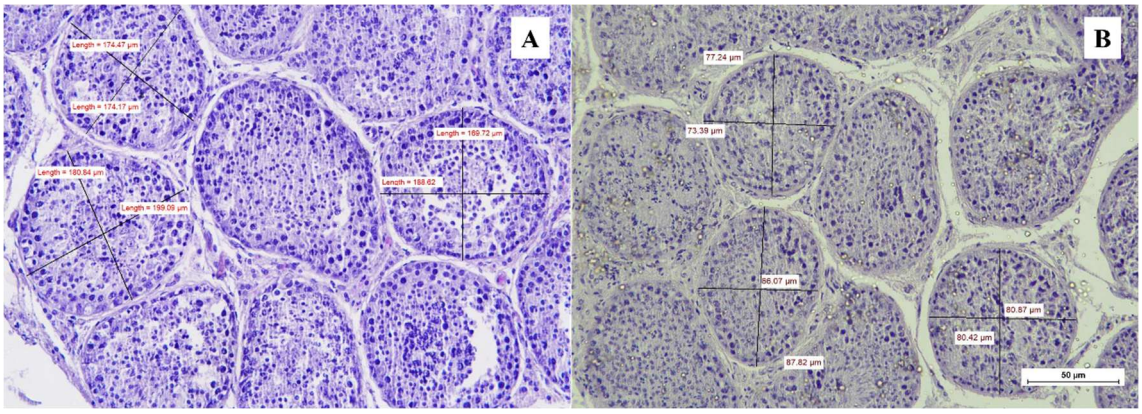

Figure 3: A. Histomorphometry of fresh canine seminiferous tubules. B. Histomorphometry of canine seminiferous tubule vitrified with ethylene glycol/glycerol association. The reduction in tubular diameter can be observed in the vitrified sample. HE staining, 400x magnification, scale 50 µm

Histomorphological analysis

Histology, a simple and cost-effective technique, has been the primary method for evaluating cellular morphology and the organization of other tissue structures. The application of this technique can be optimized by using scales to classify cellular and tissue structures, which allows for a clearer comparison between different protocols (Thuwanut et al., 2013; Yildiz et al., 2018). In studies involving testicular samples, histomorphology was used to assess the integrity of the seminiferous tubules, germ cell nuclei, Sertoli and Leydig cells (Fig. 4) (Pukazhenthi et al., 2015; Lima et al., 2017; Fernandes et al., 2021).

Viability analysis

Fluorescent probes play a fundamental role in assessing cell viability in testicular fragments. Among the most used markers in studies using testicular samples from dogs and cats, the following stand out: propidium iodide, SYBR-14, Hoechst and rhodamine 123 (Fig. 5), each with specific functions in identifying cell viability.

Propidium iodide is a fluorescent marker that is impermeable to viable or intact cells. However, it easily penetrates dead or membrane-compromised cells, and is widely used to label nonviable germ cells, emitting red fluorescence (Garner and Johnson, 1995). In contrast, SYBR-14 is a nuclear fluorescent marker that crosses the membrane of live spermatozoa, causing them to fluoresce green under an appropriate filter

(Garner and Johnson, 1995). This association allows a clear distinction between viable and nonviable cells (Buarpung et al., 2013; Pukazhenthi et al., 2015). Hoechst is a fluorescent marker that binds to DNA, labeling cell nuclei in blue. In addition to facilitating cell counting and visualization, this probe is particularly useful for detecting apoptosis, since cells undergoing programmed death present nuclear condensation and fragmentation (Carvalho et al., 2023; 2024).

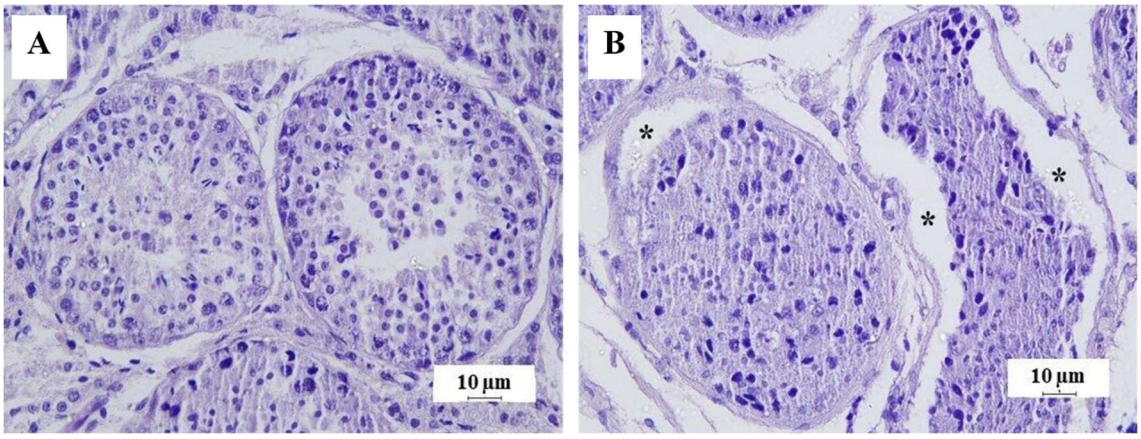

Figure 4: Histomorphological evaluation of fresh and vitrified adult canine testicular fragments. A. Fresh fragments demonstrating normal spermatogenesis; B. Vitrified fragments with dimethyl sulfoxide/glycerol association showing cellular loss and basement membrane separation (asterisks). HE vitrified, 400x magnification, scale: 10 µm.

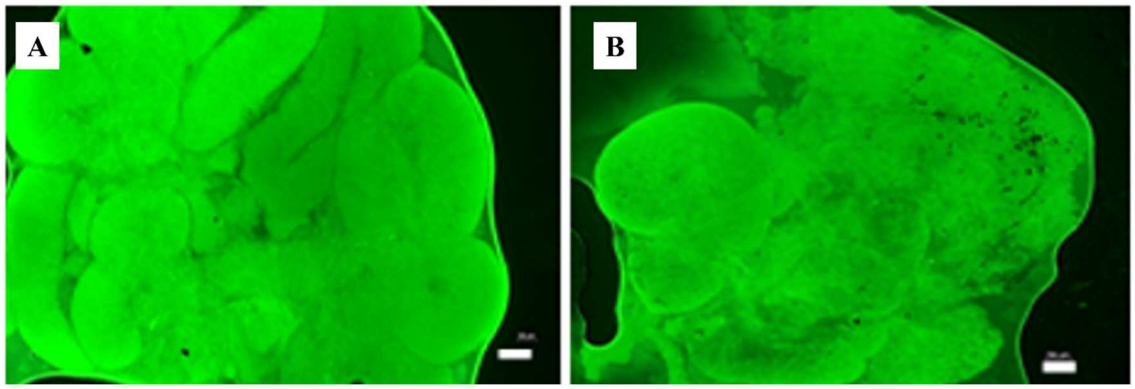

Figure 5: Photomicrographs of adult canine testicular fragments demonstrating mitochondrial activity under fluorescent microscopic analysis with Rhodamine 123. A. Fresh testicular fragment; B. Vitrified canine testes with the dimethyl sulfoxide/glycerol association showing reduction in fluorescence emission. 40x magnification, scale: 100 µm.

Another fluorophore used in studies on testicular cryopreservation is rhodamine 123. This probe allows the assessment of tissue viability through the analysis of mitochondrial membrane potential (Lima et al., 2018b; Fernandes et al., 2021). The association of these fluorescent probes provides a comprehensive approach for the analysis of cell viability and tissue integrity in cryopreserved testicular samples.

Assessment of proliferative potential

In addition to cell viability, the proliferative capacity of germ cells after cryopreservation is crucial for preserving fertility. Methods such as silver staining of nucleolar organizer regions (NORs) allows the assessment of proliferative activity by identifying proteins associated with DNA transcription and replication (Fig. 6), providing information on the growth potential of testicular cells after warming (Lima et al., 2018a; Table 2).

In vitro culture of testicular fragments has proven to be an efficient tool for monitoring the progression of cell differentiation and maintenance of germinal characteristics over time (Lee et al., 2016). Also, it is promising for the preservation of male fertility, especially when associated with cryopreservation (Yokonishi et al., 2014).

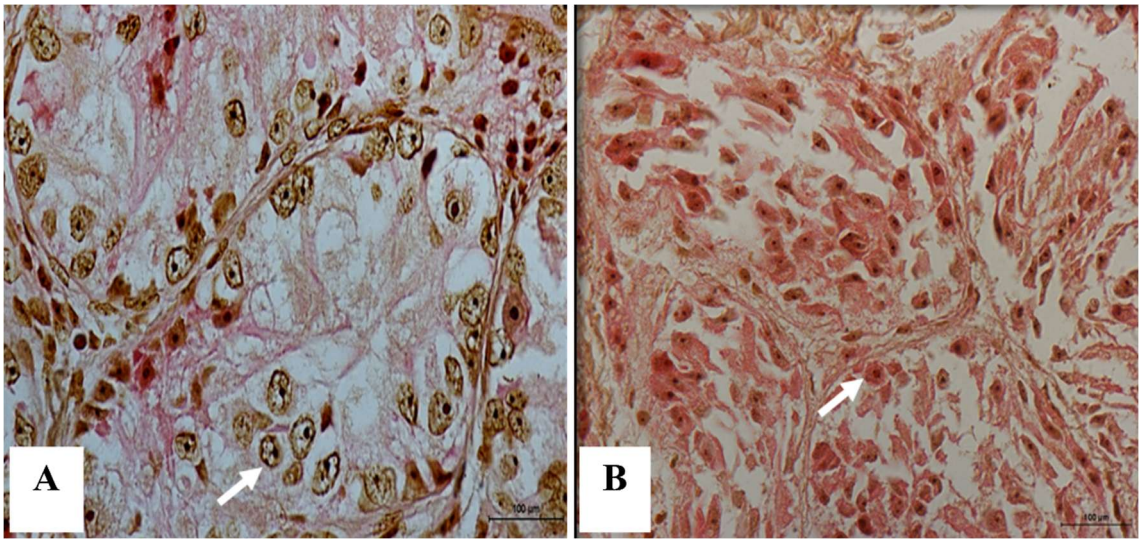

Figure 6: Nucleolar organizer regions (NORs) in spermatogonia of testicular fragments from prepubertal cats. A. Fresh fragment; B. Vitrified feline testicular fragments with dimethyl sulfoxide/ethylene glycol association showing a reduction in NORs. Arrows indicate nucleolar organizer regions in spermatogonia from testicular fragments. AgNOR, 600x magnification, scale: 100 µm.

Xenotransplantation has been an approach used to assess the development of spermatogenesis *in vivo*. This technique consists of transferring tissue between different species, allowing the analysis of cell proliferation and differentiation in an active biological environment, where host endocrine and paracrine factors can support the reactivation of spermatogenesis (Ntemou et al., 2019).

Following vitrification of testicular cells from prepubertal dogs, subsequent *in vitro* culture and subcutaneous xenotransplantation in mice led to the formation of spermatogonial stem cells, as well as the development of seminiferous tubules (Lee et al., 2016; Table 1). In cats, it was observed that testicular weight can help predict the success of xenotransplantation and define the number of mice to be used (Mota et al., 2012; Table 2). Furthermore, after a short period of culture, the pool of premeiotic germ cells returned to the same conditions as fresh material. Also, meiotic cells increased compared to the fresh group after 24 hours and maintained a high percentage after five days (Lima et al., 2018b; Table 2).

Final considerations

Testicular cryopreservation is an essential method for modern veterinary reproduction. Its continued use can significantly improve reproductive management in dogs and cats, in addition to contributing to scientific advances and genetic conservation programs.

Although the testicular cryopreservation biotechnique in dogs and cats is still in its early stages of research, significant advances have already been achieved. Traditional freezing and vitrification are described for testicular preservation, however, several factors can influence their final efficiency, which makes it unfeasible to highlight which method would be the most recommended. Therefore, it is essential to carry out new studies to deepen knowledge and establish reference parameters for testicular cryopreservation in dogs and cats.

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