Canine and Feline Testicular Preservation

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The increased interest in breeding dogs and cats and their use as models for other canids and felids demand research to improve reproductive techniques. Among them, testicular cryopreservation stands out. Testicular cryopreservation enables the maintenance of reproductive capacity and allows the establishment of germplasm banks for several species of commercial value or at risk of extinction. Furthermore, it enables the transport of genetic material among different regions. It is noteworthy that this biotechnology represents the only possibility of preserving the fertility of prepubertal animals that have died, so it has great importance in the propagation of the genetic material of animals. The spermatogonia present in the testes can be cultivated in vitro and the sperm obtained can be used in artificial reproduction programs. Although advances have been achieved with the use of testicular fragments to obtain viable and functional germ cells, the establishment of protocols that can be used in clinical routine have not been concluded yet. The testicular cryopreservation process can be carried out through techniques such as slow freezing, fast freezing and vitrification. However, the protocols used for the canine and feline species are still in the experimental phase.

dog cat testes conservation vitrification freezing

1. Introduction

Testicular cryopreservation enables the maintenance of reproductive capacity [1][2][3][4] and allows the implantation of germplasm banks for several species of commercial value or even those at risk of extinction [5]. Moreover, it provides the transport of genetic material among different regions [2]. It is noteworthy that this biotechnology represents the only possibility of preserving the fertility of prepubertal animals that have died, and it thus has great importance in the propagation of their genetic material [6].

Spermatogonia present in the testes can be cultivated in vitro, and the sperm obtained in this manner can be used in artificial reproduction programs. Although advances have been achieved with the use of testicular fragments to obtain viable and functional germ cells, the establishment of protocols that can be used in clinical routine has not been concluded yet [7].

The testicular cryopreservation process can be carried out using techniques such as slow freezing, rapid freezing and vitrification. However, the protocols used for dogs and cats are still in the experimental phase.

2. Canine Species

There are very few published works about testicular cryopreservation in dogs. The pioneer work found on this subject in dogs dates back only to 2016 [8]. The main goal of this study was to evaluate the capacity of two different cryopreservation approaches (slow freezing versus vitrification) for testicular preservation in adult dogs. **Table 1** provides a compilation of works about testicular cryopreservation in dogs.

Table 1. Papers addressing testicular cryopreservation in dogs.

Objectives	Technical Approach	Main Outcomes	Reference
Compare different cryoprotectants and compare conventional freezing with testicular vitrification.	Slow freezing and solid surface vitrification using DMSO, GLY, EG and trehalose.	Solid surface vitrification in association with DMSO and trehalose generated better results.	[8]
Evaluate the efficiency of DMSO and EG regarding the sperm membrane integrity after cryopreservation.	Slow freezing using DMSO or EG.	Both cryoprotectants were similarly efficient.	[9]
Compare two times of immersion in freezing medium and evaluate the effect on sperm membrane integrity.	Slow freezing using 15 or 30 min of immersion times.	Both the immersion times and the freezing technique used were efficient.	[10]
Compare different cryoprotectants and compare conventional freezing with testicular vitrification.	Slow freezing and solid surface vitrification using DMSO and EG isolated or in association.	Slow freezing using DMSO/EG trehalose generated better results.	[11]
Identify the optimal conditions for freezing canine testicular cells for GDCs culture, and to determine the spermatogonial stem cells capacity of these GDCs.	Slow freezing and in vivo or in vitro culture	StemPro-34 SFM as culture medium and DMSO as cryoprotectant were adequate for testis freezing, providing cells viable to develop during both in vivo and in vitro culture.	[12]
Histologically evaluate the testicles of prepubertal dogs vitrified with different associations of cryoprotectants	Solid surface vitrification using DMSO/GLY or DMSO/EG or GLY/EG	DMSO/EG and EG/GLY association were the ones that best preserved testicular integrity	[13]

3. Feline Species

DMSO: dimethyl sulfoxide; GLY: glycerol; EG: ethylene glycol; GDCs: germ-cell derived colonies.

Table 2 shows a compilation of the works found in the literature regarding testicular cryopreservation in cats.

Table 2. Papers addressing testicular cryopreservation in cats.

Objectives	Technical Approach	Main Outcomes	Reference
Focus on testis collection, cryopreservation and storage on ice-cold medium of testis fragment in an attempt to optimize conditions for posterior application to endangered felids.	Refrigeration at 4 °C or slow freezing using DMSO	Testicular cryopreservation with DMSO failed to produce grafts with germ cells. In contrast, testis from prepubertal animals may be preserved in ice cold medium for 2 to 5 days while pubertal testis showed signs of greater susceptibility to hypoxia/culture medium storage. Testis weight may be used to predict xenograft success, help decide the number of mice to use.	[14]
Determine the effects of CPAs and freezing protocols on testicular sperm plasma membrane and DNA integrity, and the fertilizing ability after ICSI	Two-step freezing vs. controlled slow freezing GLY, EG, PRO or DMSO	Testes were successfully cryopreserved. Types of CPAs and freezing techniques play a central role in determining the post-thaw quality of feline testicular spermatozoa. Frozen-thawed testicular spermatozoa retain fertilizing ability, although the development capability of embryos derived from ICSI with frozen-thawed testicular sperm is poor.	[15]
Evaluate testicular cryopreservation comparing two fragment sizes (0.3 and 0.5 cm^3) and two cryoprotectants (GLY 3% and PRO 3%).	Conventional freezing	GLY was more efficient than PRO for cryopreservation and 0.5 cm^3 fragments showed better results than 0.3 cm^3 fragments.	[16]
Evaluate the effect of different associations of cryoprotectants on testicular integrity and the potential of spermatogonial proliferation after prepubertal testicular vitrification.	Vitrification using DMSO/GLY or DMSO/EG or GLY/EG	The association DMSO/GLY showed best testicular preservation and of the potential for cell proliferation after the vitrification.	[17]
Study structural and functional properties of testicular fragments from prepubertal cats after vitrification followed by two warming protocols (directly at 37 °C or with a 5-s pre-exposure to 50 °C) and three reanimation time points (immediately, 24 h and 5 days post-warming)	Vitrification	Preservation of seminiferous tubule structure was better using warming at 50 °C/5 s, and survival of somatic as well as germinal cells was higher compared to direct warming at 37 °C/1 min. Short term in vitro culture also proved that cellular composition and functionality were better preserved when warmed for a short time at 50 °C. Short warming at 50 °C led to better quality of seminiferous tubule structure and cell composition after vitrification and short-term culture.	[7]
Evaluate the effect of different associations of cryoprotectants on the testicular integrity, the	Vitrification using cryotubes	Vitrification in cryotubes can be successfully used for the testicular cryopreservation, and the DMSO/GLY association contributed the	[18]

Objectives	Technical Approach	Main Outcomes	Reference
potential for cell proliferation, and the viability of germ cells after in cryotubes		most to the maintenance of testicular histomorphological characteristics after vitrification.	
Evaluate the damage to DNA and estimate the apoptosis rates in testicular fragments. Values for these variables were compared between type of cryoprotectant used (3% GLY and 3% PRO) and the size of the testicular fragment (0.3 and 0.5 cm^3).	Conventional freezing	Both GLY and PRO at a concentration of 3% provided protection against damage caused by cryopreservation for both sizes of fragments. However, there were differences in the efficacy of the cryoprotectants regarding protection capacity depending on the type of the cell within the tissue.	[19]
Evaluate the influence of different warming temperatures (50, 55 and 60 °C) on the structure, metabolic activity, composition, and cellular functions of the vitrified testicular fragments of pre-pubertal cats.	Vitrification using DMSO/GLY	Vitrified testicular fragments from prepubertal cats have better preserved morphology, morphometry and viability when warmed at 50 °C.	[20]

DMSO: dimethyl sulfoxide; EG: ethylene glycol; GLY: glycerol; PRO: propanediol.

questions, methods.

Anhydrobiosis is the temporary suspension of vital activities that enable an organism to tolerate long dehydration. This is a natural process used by several small organisms to resist dry conditions. It is based on the properties of trehalose, which reaches a high concentration of solute without molecular mobility during dehydration, thus avoiding intra- and extracellular degradation.

Based on the principle of anhydrobiosis, one work has been presented using the cat as a model to develop future preservation at nonfreezing temperatures. The aim of this study was to characterize changes in histology, DNA integrity, and viability of adult testis versus prepubertal individuals during microwave-assisted drying. The results demonstrated for the first time that normal morphology, incidence of degeneration, DNA integrity and viability of testis remained at acceptable levels during microwave-assisted drying for 20 min. Overall, prepubertal testis appeared to be more resilient to microwave-assisted desiccation than adult testis [21].

Although the few studies on canine and feline testicular cryopreservation have been carried out using testicular fragments, in 2020, a German team published a paper on cryopreservation of a cat testicular cell suspension. The aim of this work was to establish a cryopreservation protocol for testicular cell suspension of domestic cats to be implanted in endangered feline species, applying two concentrations of dimethyl sulfoxide (7.5 and 15%) and performing a slow and a fast freezing protocol. The best protocol was obtained with slow freezing using 7.5% dimethyl sulfoxide, resulting in a mean cell survival rate of 45.4 ± 9.1% [22].

In 2021, a published work suggested epididymal vitrification as an alternative to the conservation of the male gamete as a means of preserving individual reproductive potential. The purpose of the study was to determine the effect of the vitrification of epididymal cauda by comparing the effects of glycerol and ethylene glycol on epididymal sperm quality post-vitrification. The authors observed that epididymal tail vitrification appears to be a suitable

method for long-term storage of cat sperm, especially if the procedure is performed with ethylene glycol as the cryoprotectant [23].

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