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Testicular cryopreservation: From technical aspects to practical applications

Ana Glória Pereira, Tayná Moura Matos, Joana Letícia Cottin de Albuquerque, Andréia Maria da Silva and Alexandre Rodrigues Silva

Laboratory of Animal Germplasm Conservation, Federal Rural University of the Semiarid Region – UFERSA, Mossoró, RN, Brazil

Summary. Testicular cryopreservation has been highlighted as a promising alternative for preserving male fertility and can be applied to restore spermatogenesis in prepubertal individuals or cancer patients, preserve biologically valuable genotypes, and in studies on reproductive physiology or toxicity of various substances. This review presents an analysis of the technical aspects and applications of testicular cryopreservation, examining the contributions of important studies in this area and discussing the different factors that can impact the efficiency of the technique. Testicular fragments can be obtained from living or dead individuals, at any age and reproductive stage, through orchiectomy or biopsy. Among the methods used for processing, slow freezing and vitrification in open or closed systems stand out. However, factors such as species, age, medium used, cryoprotectants, and cryopreservation method can influence the viability of the testis after heating. To obtain sperm, the testes can be cultured *in vitro* or *in vivo* and the recovered gametes applied in assisted reproduction techniques. However, in some species, mainly wild animals and humans, this is still a limitation to be overcome.

Key words: Spermatogenesis, Biobank, Cryogenics, Fertility restoration

Introduction

Biobanks are sources of genetic resources that play a fundamental role in promoting advances in reproductive medicine and wildlife conservation (Comizzoli, 2018). Typically, most biological repositories have stored semen samples, but interest in gonadal biobanking has been growing over the years. In this sense, testicular cryopreservation has emerged as a promising technique, offering the potential for preserving male fertility, both from adults and prepubertal individuals (Perrard et al., 2016; Kaneko et al., 2019; Merlo et al., 2023).

Besides preserving male fertility (Fig. 1), testicular cryopreservation associated with culture allows us to fill the gaps in the reproductive physiology of several species, through the study of spermatogenesis and the factors that condition it (Yokonishi and Ogawa, 2016). For humans, it is also of great importance for preserving fertility in individuals undergoing cancer treatments (Younis et al., 2023). In fact, studies involving reproductive toxicology have been applied in testis to understand the gonadotoxic effects of various substances (Feraille et al., 2023).

The biggest challenge in testicular cryopreservation is to maintain cells viable after thawing, as exposing the testis to high cooling rates can lead to deleterious damage to sperm. The efficiency in obtaining sperm after warming is still limited. Thus, the establishment of appropriate protocols for each different species is essential for the success of testicular cryopreservation. For this, several methods have been employed, such as slow freezing and vitrification, which are frequently applied, but whose efficiency can vary based on several factors such as age (Amelkina et al., 2021), species (Thuwanut et al., 2013), use of cell fragment or suspension (Onofre et al., 2016; Patra et al., 2021), and warming method (Fernandes et al., 2021).

In this context, this review aims to present the technical aspects related to the efficiency of testicular cryopreservation, and discuss its main applications, exploring the contributions of relevant studies in this area.

Considerations on testicular samples

The first technical aspect to be observed concerns the origin of the sample, which may come from testicular biopsies of living individuals (Portela et al., 2019) or recovered directly from an orchiectomy or after the death of the donor animal (Thuwanut et al., 2013). It is worth noting that when using biopsies or fragments

Corresponding Author: Alexandre Rodrigues Silva, Laboratory of Animal Germplasm Conservation, Federal Rural University of the Semiarid Region – UFERSA, Mossoró, RN, Brazil. e-mail: alexrs@ufersa.edu.br
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obtained by orchiectomy, the researcher had control over the time between collection and cryopreservation of the samples; in the case of the sudden death of the individual, however, different external factors could interfere with the quality of the biological samples, such as temperature, the cause, and even the time elapsed between death and gonadal recovery (Thuwanut et al., 2013; Pothana et al., 2015). According to Tang et al. (2024), ideally, testicular samples should be stored at 4ºC for a maximum period of 24h before processing for cryopreservation since, after this period, there would be a significant increase in damage to the morphology of the seminiferous tubules, which could have negative consequences on the efficiency of the technique.

Another important aspect that can influence the success of testicular cryopreservation concerns the size of the fragment used. According to Patra et al. (2021), the use of reduced-size fragments allows faster permeation by cryoprotectants, thus reducing the cytotoxicity of these agents. In addition, there is an increase in the surface-to-volume ratio of the fragment, which facilitates greater contact with liquid nitrogen, enabling faster heat exchange, leading to better cell viability. Despite this, in the current literature, testicular fragments of various sizes have been used for different animals, ranging from 0.5 to 20 mm^3 (Lima et al., 2018a; Fayomi et al., 2019; Silva et al., 2022). In primates, large testicular fragments ranging from 9-20 mm^3 were used in cryopreservation and resulted in the production of sperm by auto-transplantation, which was applied for *in vitro* fertilization procedures, resulting in the birth of viable offspring (Fayomi et al., 2019).

In parallel to the use of testicular fragments, which are the most common, there are options of using testicular cell suspensions (TCS) or even isolated seminiferous tubules. Cryopreservation of TCS can facilitate cell survival, bypassing the obstacles of heat and mass exchange encountered with gonadal cryopreservation (Wyns et al., 2010); however, handling and cryopreservation of TCSs require exposure to high concentrations of digestive and cryoprotectant solutions that can interfere with the recovery, viability, and functionality of these cells (Onofre et al., 2016). To solve this problem, Han et al. (2021) explored the vitrification of isolated seminiferous tubules through an innovative cryoprotectant microinjection technique, which maintained the structural integrity of the tubule and the number of germ cells, also reducing spermatogonial apoptosis after cryopreservation in comparison with vitrification without microinjection (Han et al., 2021). According to Han et al. (2021), isolated seminiferous tubules are more suitable for vitrification than larger sections because larger volumes may lead to incomplete penetration of cryoprotective

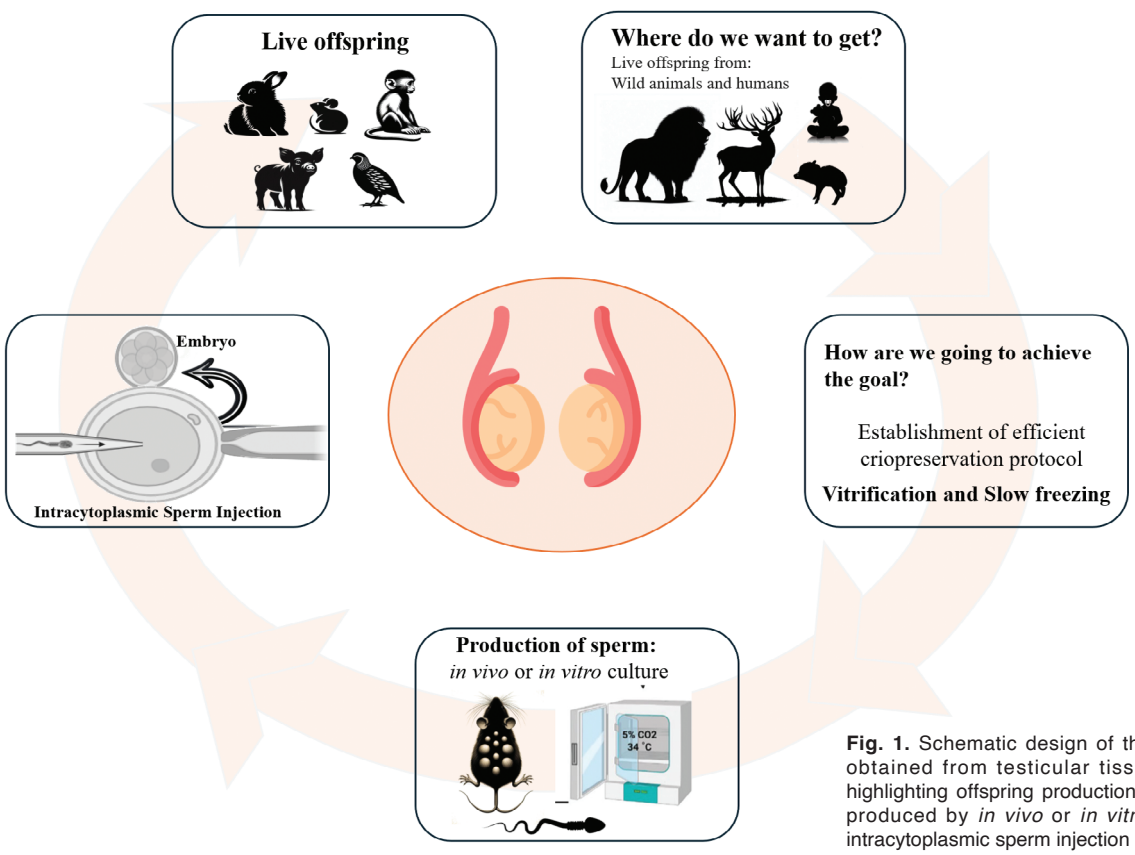

Fig. 1. Schematic design of the main achievements obtained from testicular tissue cryopreservation, highlighting offspring production derived from gametes produced by *in vivo* or *in vitro* culture followed by intracytoplasmic sperm injection (ICSI).

agents, and slower cooling and thawing rates may lead to incomplete vitrification, resulting in ice crystal formation and sample damage.

The age of the animal donating the testicular sample is another important factor to consider. Amelkina et al. (2021), when comparing the effects of testicular vitrification from prepubertal and adult cats, revealed that cryopreservation caused more detrimental effects on adult samples than on immature juveniles. In another study in the same species, it was found that samples from prepubertal individuals were more resilient to microwave-assisted dehydration for anhydrous preservation (Silva et al., 2020a). When considering the application of testis cryopreserved *in vivo* or *in vitro* culture, the low spermatogenic activity of prepubertal testis has been shown to be a factor contributing to successful testicular survival and development (Sato et al., 2015).

Media and cryoprotective agents

Choosing an appropriate medium is essential for the efficiency of the cryopreservation protocol since the medium provides the nutrients, pH, and osmotic environment suitable for the testis during the procedure (Nema and Khare, 2012). It is then necessary to select an appropriate medium for each type of fragment or cell or, when necessary, to promote modifications in the medium so that it adapts to the nature of the material to be preserved (Yao and Asayama, 2017).

Different media have been associated with testicular cryopreservation, such as minimum essential medium (MEM) (Silva et al., 2022), α-minimum essential medium (α-MEM) (Yokonishi et al., 2014), Dulbecco's modified Eagle's medium (DMEM) (Singh et al., 2019), DMEM-f12 (Amelkina et al., 2021), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS) (Thuwanut et al., 2013), and modified North Carolina State University (NCSU)-37 solution (Kaneko et al., 2017). Despite the diversity, most studies usually address the comparison of the cryopreservation medium, which is a combination of the base medium and cryoprotectants, also including additional protein sources such as fetal bovine serum or bovine serum albumin (Yokonishi et al., 2014; Silva et al., 2021). Some of the additional protein sources have cryoprotective functions for cells or fragments (Wu et al., 2014), acting in conjunction with cryoprotectants (Skorupa and Piasecka-Belkhayat, 2023).

Cell survival through the rigors of freezing and thawing in cryopreservation procedures is only achieved by using an appropriate cryoprotectant. These are chemical compounds specifically designed to protect biological samples from damage during freezing when used at the appropriate concentration (Skorupa and Piasecka-Belkhayat, 2023). Among the penetrating cryoprotectants, dimethyl sulfoxide (DMSO; molecular weight 78 g/mol) has stood out for presenting promising results in the testicular preservation of different species such as the Iberian lynx (*Lynx pardinus*) (Leon-Quinto et al., 2009) and the spotted tragulum (*Moschiola indica*) (Pothana et al., 2015). Its use has even resulted in the production of sperm and, consequently, the birth of viable offspring in mice, rabbits (Shinohara et al., 2002), and Rhesus monkeys (Fayomi et al., 2019). To minimize the possible toxic effects of isolated cryoprotectants, their use in combination has been suggested. Several studies, for example, show the efficiency of a combination of DMSO and ethylene glycol (EG) in cryopreserving testicular fragments of collared peccaries (*Pecari tajacu*) (Silva et al., 2021), Persian leopard (*Panthera pardus saxicolor*), pygmy goat (*Capra aegagrus hircus*), capuchin monkey (*Cebus apella*), Patagonian mara (*Dolichotis patagonum*) (Merlo et al., 2023), and domestic dog (Teixeira et al., 2021).

The combination of penetrating and non-penetrating cryoprotectants, such as sugars and polyvinylpyrrolidone (PVP), has also been shown to be effective for testicular cryopreservation (Kaneko et al., 2013, 2017). Sugars, such as sucrose and trehalose, protect cell membranes against cold-related injury through their connection with phospholipid head groups present in the membrane (Dhaliwal et al., 2019). On the other hand, PVP is a water-soluble polymer made of N-vinylpyrrolidone with high molecular weight, which acts as a non-penetrating cryoprotectant and has been shown to protect various nucleated cells of vertebrates (Damjanovic and Thomas, 1974; El-Shewy et al., 2004). The use of trehalose, PVP, and EG as cryoprotectants has proven useful for ultrafast cooling of sperm-producing pig fetal testis in xenograft (Kaneko et al., 2017) and live birth of piglets (Kaneko et al., 2013).

Cryopreservation methods

Methodologies for processing testicular samples may vary depending on the animal species that donated the sample and the research groups (Yokonishi et al., 2014; Silva et al., 2022). In general terms, cryopreservation methodologies (Fig. 2) could be grouped as slow freezing or vitrification methods, as exemplified in Table 1 for different species.

Slow freezing uses a low cooling rate and low-concentration cryoprotectant solutions and is considered to have low cytotoxicity to cells; however, the risk of cell injury due to ice crystal formation is high (Xu et al., 2010). To perform this procedure, the cryopreservation solution is placed inside a cryovial where the fragments are immersed. Each cryovial is then processed directly in an automated freezing machine using an appropriate curve (Milazzo et al., 2008). Another possibility would be to place the cryovial in a Nalgene-type container (Mr. Frosty®, Thermo Fisher Scientific, Wilmington, United States) containing isopropyl alcohol at 25°C, which would then be transferred to a -80°C freezer overnight (Singh et al., 2019; Andrae et al., 2021). The technique has been extensively used in several species and has already enabled the birth of live offspring in mice,

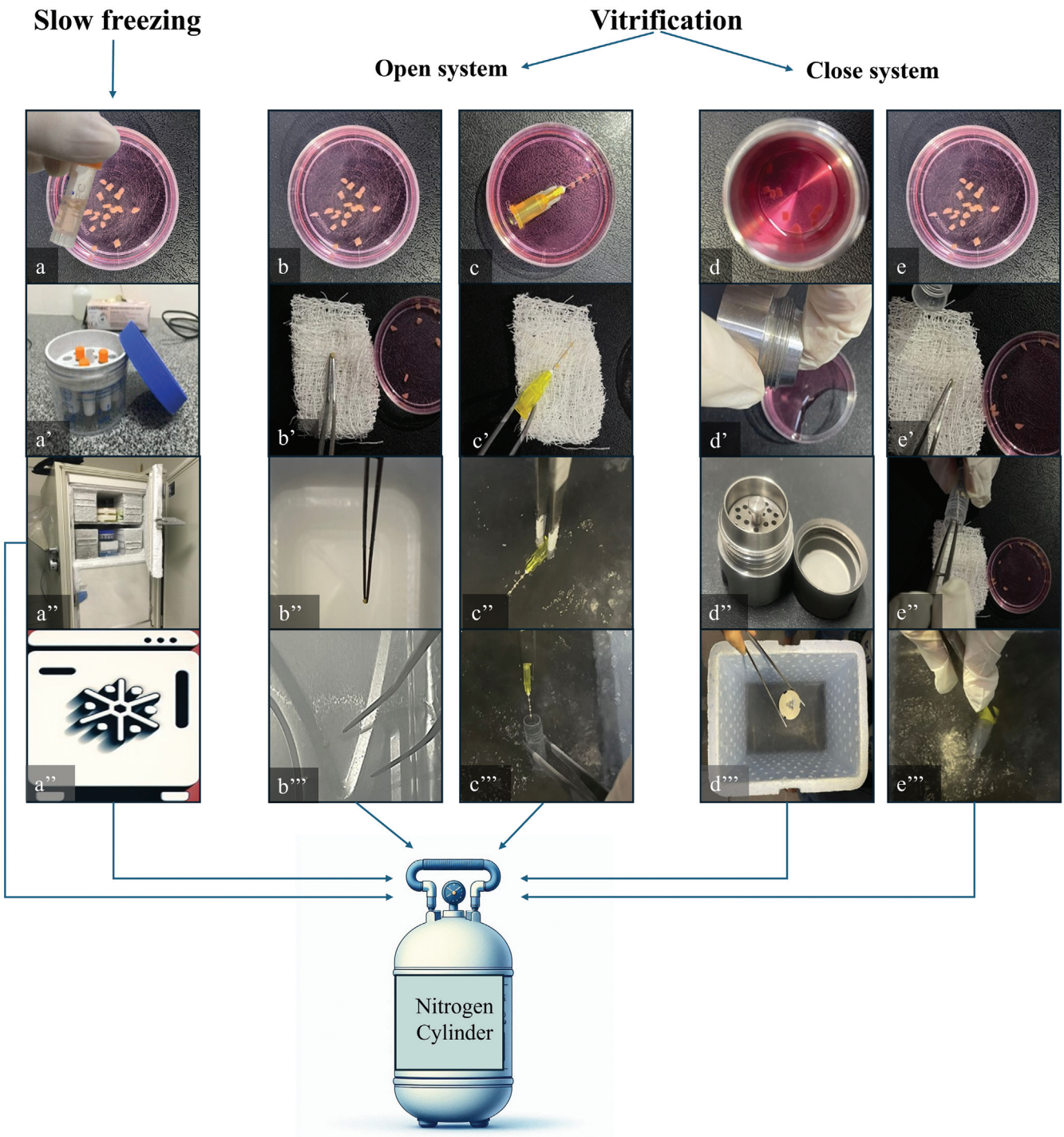

Fig. 2. Main cryopreservation protocols. **(a, b, c, d, and e)**. The testis fragments are submerged in cryopreservation solution. For the slow freezing method, samples are stored in cryotubes **(a')** and stored for 12 hours in a freezer at -80 °C **(a'')** or using a freezing machine **(a''')**. The vitrification method can be conducted in both open or closed systems, but samples are first immersed in vitrification solution **(b, c, d, and e)**, and the excess solution is removed **(b', c', d', and e')**. The fragments can be placed on a solid surface (SSV) in contact with the nitrogen **(b")**, directly submerged in nitrogen **(c")**, kept inside the device **(d")**, or placed inside the cryotube **(e")**. In the open system, the fragments are placed in a cryotube after vitrification **(b''' and c''')**; in the closed system, testis fragments are placed in the dispositive or cryotube before they are submerged in liquid nitrogen. Finally, all samples are stored in a nitrogen container.

rabbits (Shinohara et al., 2002), and rhesus monkeys (Fayomi et al., 2019).

Vitrification is the process in which a liquid solidifies without forming crystals. This occurs in systems that are highly concentrated or have a very rapid cooling rate (from -20,000 to -40,000_C/min), causing the viscosity to increase rapidly and preventing the molecules from arranging themselves into a crystalline structure (Wowk, 2010). As cooling continues, the viscosity increases to the point where molecular movement decreases significantly, transforming the liquid into a glass. The final solid maintains the random molecular arrangement of the liquid but has the mechanical properties of a solid (Wowk, 2010).

In open vitrification systems, the fragments are previously immersed in an equilibrium solution (ES), usually for 10 minutes, and then in the vitrification solution (VS) for 3 to 5 minutes; although there are protocols in which ES is not used, and the samples come into direct contact with the VS (Silva et al., 2022). Subsequently, the testicular fragments are vitrified on a surface in contact with nitrogen or immersed directly in liquid nitrogen and then placed in a cryovial and stored in a nitrogen container (Silva et al., 2021, 2022). Through these procedures, however, it is worth noting that the exposure of testicular fragments to direct contact with liquid nitrogen can determine risks of potential cross-contamination, as well as the transmission of diseases when considering long-term cryopreservation (Joaquim et al., 2017).

There are two most common methods for conducting testicular vitrification in open systems. In the first, called

Table 1. The main achievements from testicular cryopreservation using slow freezing and open solid surface - SSV and needle immersion (NIV) or closed (cryotubes) vitrification systems.

Systems	Animal	Medium	Cryoprotectants	Main results	References
Slow freezing	Grey wolf (*Canis lupus*) (Adult)	DMEM	7.5% EG; 7.5% DMSO; 20% FBS	Preservation of morphology, thus supporting cell viability.	Andrae et al., 2021
	Rhesus Monkey (immature)	α-MEM	5% DMSO	Production of live offspring	Fayomi et al., 2019
	Sheep (immature)	DMEM	20% DMSO; 20% FBS	Production of round and elongated spermatids	Pukazhenthi et al., 2015
	Transgenic mice (immature)	Cell Banker	--	Production of live offspring	Yokonishi et al., 2014
	Pig (immature)	DMEM	7% Glycerol; 5% FBS	Production of round and elongated spermatids	Abrishami et al., 2010
	Human	HBSS	0.7 M DMSO; 0.1 M sucrose; 10 mg/ml HSA	Spermatogonia survival after cryopreservation and transplantation	Wyns et al., 2008
	Mouse and Rabitt (immature)	DMEM	DMSO; FCS	Production of live offspring	Shinohara et al., 2002
Vitrification Open					
SSV	Meishan pig (immature)	IVC-PyrLac solution	VS: 35% EG, 5% PVP; 0.3 M trehalose	Production of embryos derived from spermatozoa obtained after culturing vitrified samples	Kaneko et al., 2019
SSV	Mice (immature)	DMEM	15% DMSO; 15% EG; 20% FBS; 0.5 M sucrose	Production of sperm	Dumont et al., 2015
SSV	Transgenic mice (immature)	Stem Cell Keep	--	Production of live offspring	Yokonishi et al., 2014
SSV	Pig (immature)	NCSU-37	VS: 35% EG, 5% PVP; 0.3 M trehalose	Production of live offspring	Kaneko et al., 2013
NIV	Pig (immature)	DMEM	VS: 15% DMSO; 15% EG; 20% FBS; 0.5 M sucrose	Production of round and elongated spermatids	Abrishami et al., 2010
NIV	Grey wolf (*Canis lupus*) (adult)	DMEM	15% EG; 15% DMSO; 20% FBS; 0.5 M sucrose	Maintaining of cell viability after thawing	Andrae et al., 2021
	Japanese Quail (*Coturnix japonica*)	HM	15% EG; 15% DMSO; 0.5 M sucrose	Production of live offspring	Liu et al., 2013
Closed					
Vitrification in cryovial	Mouse	α-MEM	20 % DMSO; 5% GelMA	Improved morphology, mitochondrial activity, and enhanced antioxidant capacity	Tang et al., 2024

DMEM, Dulbecco's Modified Eagle Médium; DMSO, Dimethylsulfoxide; EG, Ethyleneglycol; FBS, Fetal Bovine Serum; PVP, Polyvinylpyrrolidone; GelMa, Gelatin-methacryloyl.

solid surface vitrification (SSV), the samples are exposed to an aluminum foil, a solid surface (with high thermal conductivity), in contact with liquid nitrogen (Kaneko et al., 2013; Silva et al., 2021). In the second, the testicular fragments are pierced by a 30G needle, facilitating their direct exposure to liquid nitrogen (NIV) (Liu et al., 2013; Fernandes et al., 2021; Picazo et al., 2022; Merlo et al., 2023). In general, both methods are efficient since the use of SSV for testis has already enabled the birth of pig offspring (Kaneko et al., 2013), while NIV enabled the birth of Japanese quail (*Coturnix japonica*) offspring from cryopreserved testicular samples (Liu et al., 2013).

Closed systems can avoid direct contact with liquid nitrogen and, consequently, testicular contamination during vitrification (Silva et al., 2022). In this system, the fragments are placed in a closed device before cryopreservation (Singh et al., 2019; Andrae et al., 2021; Carvalho et al., 2023). Generally, the fragments are also exposed to ES and VS solutions and then deposited inside a cryovial, which is directly immersed in liquid nitrogen and subsequently stored in a nitrogen container (Yokonishi et al., 2014; Lima et al., 2018b). Through this system, the most prominent results were found in mice, with the generation of live descendants from cryopreserved testicular fragments (Yokonishi et al., 2014).

Warming temperature

To result in effective sperm production after cryopreservation, all factors discussed here need to be taken into account, including sample warming. Testis can be cryopreserved for an indefinite period, but this is a critical point. During heating, germ cells encounter toxic and stressful conditions, including exposure to cryoprotectants and osmotic shock, which can reduce cell viability (Silva et al., 2021). Therefore, it is crucial to carefully control the time and temperature during the warming process to minimize cell death (Lima et al., 2018a). For that, different temperatures ranging from 37 to 50°C have been used, but their results may vary according to the age of the donor, considering adult or immature individuals (Fernandes et al., 2021; Macente et al., 2022).

Studies on spermatogenesis

The testicle maintains an elaborate communication between the various cell types and subpopulations that cooperatively drives the differentiation of male germ cells into haploid gametes and the production of androgens through the process of spermatogenesis (Pendergraft et al., 2017). Spermatogenesis is an essential process for understanding the physiological patterns that can elucidate assisted reproduction protocols, being a complex process that requires a suitable germ cell environment that can be impaired after warming and resuscitation (Lima et al., 2018b). This phenomenon, however, is not yet well understood for most species, especially wild ones, given the great biological biodiversity. In this sense, when starting a program focused on the use of testicular cells or fragments of a given species for the formation of biobanks, it is often necessary to carry out basic work to elucidate its spermatogenic process. The information generated by these studies is crucial, especially in the application of analysis methodologies that will attest to the efficiency of testicular cryopreservation procedures.

To this end, spermatogenesis has been studied in detail in several species. A good example is the studies conducted in collared peccaries (Costa et al., 2010), in which a very peculiar testicular architecture was characterized by the presence of isolated niches of Leydig cells forming cords in the interstitial middle. This cytoarchitecture was also observed after the transplantation of testicular cells isolated from immature testis of peccaries into immunodeficient mice, where after 8 months of culture, spermatogenesis was reestablished along with this particular structural arrangement, revealing that this pattern is important for the progression of spermatogenesis in the species (Campos-Júnior et al., 2014). Knowledge of these peculiarities in testicular architecture was crucial for establishing effective cryopreservation (Silva et al., 2020b; 2021) and testicular culture (Silva et al., 2024) methodologies for the species.

In parallel with basic histological studies, attempts to establish culture systems for fresh or cryopreserved testis have contributed greatly to the elucidation of the regulatory mechanisms of spermatogenesis. In general, the supplementation of the *in vitro* testicular culture medium has been investigated by supplementing it with growth factors, nutrients, and hormones (Ibtisham et al., 2023; Silva et al., 2024). In this sense, it is known that a protein source supplement is important for the metabolic nutrition of testicular cells during culture. Therefore, a study involving the use of Fetal Bovine Serum (FBS) and KnockOut Serum Replacement (KSR) in the *in vitro* culture of testicular fragments from prepubertal rats showed that samples cultured in medium supplemented with KSR were able to sustain growth and gradually increase the diameter of the seminiferous tubule during the culture period. In addition, spermatogonia, primary spermatocytes, secondary spermatocytes, and round spermatids were cultured after 4 weeks of culture (Liu et al., 2016). The authors justify that FBS does not have a well-defined composition and may contain factors that induce cell death, while KSR, being well-defined, would eliminate possible uncertainties. Both have already been tested in swine testicular cell culture (Fayaz and Honaramooz, 2022), where samples cultured with FBS or a combination of 5% FBS + 10% KSR led to greater gonocyte development than with the isolated use of KSR, revealing that the choice of protein source to be used in the supplementation of the culture medium may vary according to the species.

Culture systems also help identify growth factors

that contribute to the progression of spermatogenesis. When evaluating the effect of supplementing the culture medium of testicular fragments of prepubertal pigs with the growth factors GDNF, bFGF, SCF, and EGF for 8 weeks, Ibitsham et al. (2023) found that supplementation with GDNF and bFGF maintained better morphological integrity and number of gonocytes and induced spermatogenesis *in vitro*, obtaining spermatocytes. In collared peccaries, Silva et al. (2024) analyzed the effects of the growth factor GDNF and the type of base medium (DMEM or StemPro-34) on the *in vitro* culture of testicular fragments of prepubertal collared peccaries, where the addition of 10 ng/ml of GDNF to both tested media was efficient in maintaining cell survival and control.

In addition, *in vitro* studies also elucidated the role and importance of hormones in spermatogenesis, such as the isolated role of follicle-stimulating hormone (FSH) or in synergy with testosterone to prevent cell apoptosis and restore spermatogenesis (Vigier et al., 2004; Tajik et al., 2014). In fact, the use of FSH combined with insulin-like growth factor I (IGF-I) in the *in vitro* culture of Sertoli cells isolated from calves can promote increased cell proliferation after 8 days of culture (Dance et al., 2017). In the culture of spermatogonia and Sertoli cells isolated from prepubertal dogs (Pieri et al., 2019), FSH promoted an increase in spermatogonial stem cells positive for pluripotency markers when compared with samples cultured in the absence of this hormone, highlighting that FSH can promote specificity and increase the self-renewal rate of these cells.

Conservation of reproductive potential

The conservation of reproductive potential in animals and humans is considered a demand and a necessity, and reliable and sustainable germplasm storage is increasing exponentially (Comizzoli et al., 2022). In this sense, several studies on testicular technology have been developed to establish protocols for conserving the genetic heritage of endangered animals (Silva et al., 2020b) and humans (Younis et al., 2023). For both species, however, after the application of testicular cryopreservation, it is necessary to complete the spermatogenesis process (Kaneko et al., 2019). To mitigate the challenges, the main experimental approaches used were testicular germ cell or fragment transplantation to host animals (Kanatsu-Shinohara, 2022; Kanbar et al., 2022) or *in vitro* culture of testicular germ cells or fragments (Saulnier et al., 2021; Wang et al., 2022). In fact, cryopreservation of testes in conjunction with testicular xenograft can be the most effective way to preserve the germplasm of various species (Pukazhenthi et al., 2006) since the results of *in vitro* culture are still limited for obtaining sperm in different species. Thus, through the intracytoplasmic injection method, sperm obtained can be used in embryo production, promoting the generation of offspring from biologically interesting genotypes (Yokonishi et al., 2014). Unfortunately, so far, the most impressive results have been directed at immature individuals (Silva et al., 2019).

The most impressive results obtained using testicular cryopreservation technology have undoubtedly been obtained in laboratory animals. Yokonishi et al. (2014) obtained complete spermatogenesis from testicular fragments of immature mice subjected to vitrification or slow freezing, obtaining sperm production from *in vitro* culture, followed by microinjection into oocytes, generating four offspring. In prepubertal rhesus monkeys, testicular cryopreservation associated with autologous transplantation was able to promote complete spermatogenesis; the recovered sperm were used for intracytoplasmic injection, thus producing embryos that were transferred to recipient females. After the gestational period, the birth of a healthy female was confirmed (Fayomi et al., 2019).

In domestic animals, testicular cryopreservation associated with *in vitro* culture or xenograft has been efficient in obtaining male gametes, especially in production animals. In prepubertal pigs (Abrishami et al., 2010) and fetuses (Kaneko et al., 2017), cryopreservation of testicular fragments followed by xenotransplantation in mice ensured the resumption of spermatogenesis and the production of sperm. In prepubertal goats, *in vitro* culture of testicular fragments that were subjected to vitrification on a solid surface resulted in the formation of elongated sperm (Patra et al., 2021). On the other hand, the great diversity of species and the lack of information on reproductive physiology and, mainly, on the mechanisms that control spermatogenesis in wild animals hinder the advancement of this technique. Thus, although studies using fresh material have already demonstrated the ability to resume complete spermatogenesis *in vitro* in marmosets (Schlatt et al., 2002), bison (Abbasi and Honaramooz, 2011), white-tailed deer (Abbasi and Honaramooz, 2012) and collared peccaries (Campos-Junior et al., 2014), the *in vitro* development of spermatogenesis from cryopreserved testicular has not yet been achieved for wild species. One of the main studies ever conducted with wild species was reported by Silva et al. (2024), who evaluated the influence of different media and different concentrations of glial cell line-derived neurotrophic factor (GDNF) during testicular *in vitro* culture of prepubertal collared peccaries and highlighted the efficiency of DMEM and StemPro-34 SFM media supplemented with GDNF at a concentration of 10 ng/ml in maintaining cell survival and proliferation.

It is worth noting that all studies that obtained viable sperm through testicular culture used immature individuals. Previous attempts in mature wild animals resulted in cellular degeneration in the Iberian lynx, Cuvier's gazelle, and Mohr's gazelle (Arregui and Dobrinski, 2014). In adult rats and hamsters, signs of severe atrophy were observed after *in vivo* culture of testicular fragments. Furthermore, compared with immature fragments cultured in the same species,

restoration of spermatogenic function was markedly lower in mature samples (Schlatt et al., 2002). Some factors, such as delayed angiogenesis, greater cellular diversity, cytoplasmic loss in differentiated cells, and low proliferative potential of germ cells, may be associated with this degeneration (Arregui et al., 2008).

In humans, several studies have investigated cellular mechanisms regarding clinical applications of testicular cryopreservation technology due to the increasing number of patients with infertility caused by cancer therapy (Silvestris et al., 2020; Cavaliere et al., 2021; Medenica et al., 2022). Although complete spermatogenesis has not yet been achieved, Younis et al. (2023) demonstrated that testicular fragments from immature cancer patients could be cryopreserved and cultured in an organotypic system *in vitro* for 32 days, enabling the development of spermatogonia to spermatocytes. These promising results have encouraged researchers to invest in this technology to improve cryopreservation and culture protocols for testicular fragments. Alternatively, it has been shown that prepubertal individuals who have undergone chemotherapy or radiotherapy for cancer or other conditions can restore their fertility by isolating cells from biopsied testicular samples and cryopreserving them (Picton et al., 2015). For future resumption of spermatogenesis, these cells can be transplanted into the seminiferous tubules of the donor testis after treatment is completed, or they can be cultured *in vitro* or transplanted into the subcutaneous tissue of mice (Patra et al., 2021). Indeed, studies using rodent models have shown that cryopreserved testicular stem cells can colonize the empty seminiferous tubules of the recipient testes to produce spermatogenesis for the donor and restore male fertility (Kanatsu-Shinohara et al., 2003; Wu et al., 2012).

Toxicology

In parallel with the development of methods for testicular cryopreservation, there is a need to establish efficient culture protocols to make the biobank usable. As a consequence, the study of toxicology in testicular cell culture has become a new and very important area within reproductive biology. In the past year, the use of cell culture technology has increased in several sectors, such as organ disease modeling (Hidalgo Aguilar et al., 2022), organ development (Zheng et al., 2021), drug screening (Law et al., 2021), and toxicology testing (Sun and Ma, 2023).

The establishment of culture systems for *in vitro* spermatogenesis allows experiments that would otherwise be difficult to perform directly *in vivo*, such as pharmaceutical or toxicological studies of new drugs or potential toxicants in spermatogenesis (Ibtisham and Honaramooz, 2020), resulting in improvements in the interpretation of data obtained from *in vivo* systems. Furthermore, cell cultures allow toxicological tests to be performed on increasingly specialized groups of cells. The closer the model is to human physiology, the more likely the results will be relevant (Pamies and Hartung, 2017).

Several substances can impair male reproduction. To clarify the mechanisms of their action on gonads, *in vitro* and *in vivo* studies were performed to elucidate how toxic substances affect the production of fully functional sperm (Rahman et al., 2021; Vassal et al., 2021; Yuan et al., 2024). To study the impact of different molecules, some authors have employed plate culture (2D) of specific populations of germ and somatic cells. For example, Tremblay and Delbes (2018) conducted an experiment to determine whether doxorubicin can induce oxidative stress in spermatogonia (GC-6Spg) and immature Sertoli cell lines (Ser-W3). The authors used an immature Sertoli cell line isolated from prepubertal rats in a culture system in DMEM and concluded that doxorubicin induces time- and dose-dependent cytotoxicity in these two cell lines, with the Ser-W3 cell line being more sensitive than GC-6Spg. Another interesting example is the study conducted by Karmakar et al. (2018), also using the 2D-culture model to test the effect of chemotherapeutic drugs, such as etoposide, cisplatin, bleomycin, and their combination (BEP), on the physiology and activity of mouse germ stem cells *in vitro*. The authors concluded that at specific concentrations (0.05 μM etoposide, 1 μM cisplatin, 10 μM bleomycin, and 0.1 μM BEP), the drugs affect the viability of germ cells and significantly decrease their proliferative capacity.

On the other hand, some authors prefer to culture testicular cells in other models of previously validated culture systems, such as the 3D-culture system (Sato et al., 2013; Dumont et al., 2015; Reda et al., 2016). In this system, testicular cells are inserted between two gels of different gradients, seeking to maintain the architecture and specific interactions of the testis, providing an accessible *in vitro* model to help better understand how germ cells interact in their environment (Sakib et al., 2019). As an example, Sychrová et al. (2022) conducted a study that compared the 2D model with the 3D model and characterized an *in vitro* 3D model of prepubertal murine Leydig TM3 cells in a toxicological assay of endocrine-disrupting chemicals. The authors concluded that the scaffold-free 3D model allowed prepubertal Leydig TM3 cells to be cultured under more physiologically relevant conditions. They stated that the 3D system maintains cell viability and stable characteristics that improve the expression of genes critical for steroidogenesis when compared with 2D cultures. In this sense, drug and toxicity screening in such 3D models would provide more physiologically relevant readouts than 2D-culture modalities (Chapin et al., 2013). Therefore, the culture format shapes cellular responses to drugs and defines the translational power of a drug assay (Jabs et al., 2017).

In addition to the culture models already mentioned above, Allen et al. (2020) suggest the use of an organotypic model with a six-day culture duration. The authors used this model with the aim of comparing the

gonadotoxic impact of the chemotherapy drugs cisplatin and carboplatin on prepubertal gonads of female and male mice and concluded that at equivalent concentrations in patients, carboplatin is no less gonadotoxic than cisplatin.

Finally, it is worth noting that different cell types can be isolated and genetically altered or exposed to environmental factors. These cells can then recombine to produce organoids that exhibit specific disease characteristics, as in the case of testicular cancer cells, which can be programmed to generate cultures of testicular malignancy. Thus, such models would be invaluable for early testing of pharmaceutical and chemotherapeutic interventions (Sakib et al., 2019).

Final considerations

The main objective of testicular cryopreservation is to obtain viable germ cells for the generation of future offspring. Testicular cryopreservation, combined with gonadal transplantation techniques such as xenograft and culture, can be an effective solution to reduce genetic loss in animals that have died before the establishment of their sperm population. In humans, it can also be used for patients who need to undergo gonadotoxic treatments.

The success of spermatogenesis from cryopreserved testicular samples depends on the number of live germ cells after heating. In addition, nuclear DNA must be intact, and apoptosis must be avoided to ensure fertility preservation. Preventing ice recrystallization during heating procedures is essential to preserve the quality of biological components present in testes. With cell structures intact after cryopreservation, spermatogenesis can be resumed, and sperm can be obtained for future use.

There is no ideal cryopreservation protocol that can be applied to all species. Each species has its particularities regarding reproductive physiology and sensitivity to testicular cryopreservation. Studies of different cryopreservation techniques, as well as concentrations of cryoprotectants should be employed for each species to determine the most appropriate protocol.

Additionally, the application of testicular culture systems associated with the cryopreservation protocols enables not only the progress of spermatogenesis but also provides a relatively unlimited number of spermatozoa to be used in other biotechnologies. In addition, culture systems generate knowledge about the reproductive physiology of the species involved and promote a controlled microenvironment for the maintenance of the microstructure of the testicular fragment. This allows the understanding of events at the molecular level that affect spermatogenesis since it permits the verification of the isolated action of different factors in spermatogenesis. However, studies on the conditions of cultivation and cryopreservation, culture media, and supplementation are still needed to ensure success in obtaining spermatozoa, especially in adult individuals.

Despite this, these techniques are still in the experimental stage for humans. *In vitro* approaches focus on the generation of male germ cells from the direct maturation of germ cells in various culture systems, along with human induced pluripotent stem cells and embryonic stem cells. These approaches mark significant advances in understanding and promoting spermatogenesis, but achieving fully functional sperm *in vitro* remains a challenge.

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