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Cryopreservation of Preantral Follicles

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Abstract

In mammals, the total number of female germ cells is already established by the time of birth, meaning that no mitosis will take place in oogonias thereafter. Their cryostorage, therefore, depends on ovarian tissue manipulation. As an alternative to mature oocyte cryopreservation, the maintenance of inactive preantral follicles is a remarkable option because (i) their availability in the ovary is greater; (ii) as inactive and small structures, they show less sensitivity to cryoinjury and the toxic effects of cryoprotectants; and (iii) they are present in the gonads at all ages, allowing their retrieval from prepubertal individuals or even immediately *postmortem*. Nevertheless, some difficulties remain regarding their *in vitro* activation and development to the ovulatory stage. For this reason, the best option for their total development is transplantation back to the donor or between species, promoting follicle activation and development. This technique has proved its efficiency and led to several live births in both animals and humans. Since each species has its own particularities in terms of ovarian tissue composition, a number of protocols have been documented, which may be used for either isolated or *in situ* preantral follicles.

Keywords: cryostorage, fertility preservation, germ cells, isolated follicles, oocyte, ovarian tissue

1. Introduction

In 1866, interest in storing human male germ cells from individuals no longer able to mate was proposed, for the first time, suggesting the possibility of generating cryobanks [1]. However, interest in the storage of female genetic sources emerged only in the 1950s with the first signs of successful cryopreservation in mice published in 1958 [2]. Since then, this strategy has spread through different species, and advances have been made in a great variety of animals.

While male germ cells are isolated in seminal fluid, their female counterparts are contained in a specific structure—the ovarian follicle. These follicles consist of an oocyte surrounded by one or two types of cells, granulosa and theca cells, which have supporting and steroidogenic functions. Ovarian follicles are generally classified as preantral and antral, depending on the presence of fluid around the granulosa cells. Preantral follicles are subclassified into primordial, primary, and secondary. These small structures form the vast majority of available mammalian oocytes and are the largest source of female genetic material [3]. When primordial follicles are activated and commence their growth, granulosa cells alter their morphology. This is the first sign of follicle development, followed by proliferation of the granulosa cells, oocyte growth, formation of the zona pellucida, changes in oocyte organelles, development of theca cells, and accumulation of follicular fluid. Eventually, a follicle may reach the preovulatory stage when ovulation occurs, releasing the oocyte ready for fertilization (**Figure 1**).

Because oocytes within preantral follicles are smaller, less differentiated, and almost metabolically inactive, they are more resistant to possible damage caused by cryopreservation procedures [4]. This is one of the reasons why so much interest has been shown in their cryopreservation as an alternative to fully grown oocytes, which are usually collected from large antral follicles. In addition, it is known that cryopreservation of cumulus-oocyte complexes (fully developed oocytes surrounded by cumulus cells obtained from antral follicles) or mature oocytes may be difficult in some species as their membrane has a low permeability coefficient with respect to cryoprotectants (CPAs) [5]. Even worse, the cryopreservation procedure may lead to zona pellucida hardening, which could hamper fertilization [6]. Since oocytes in

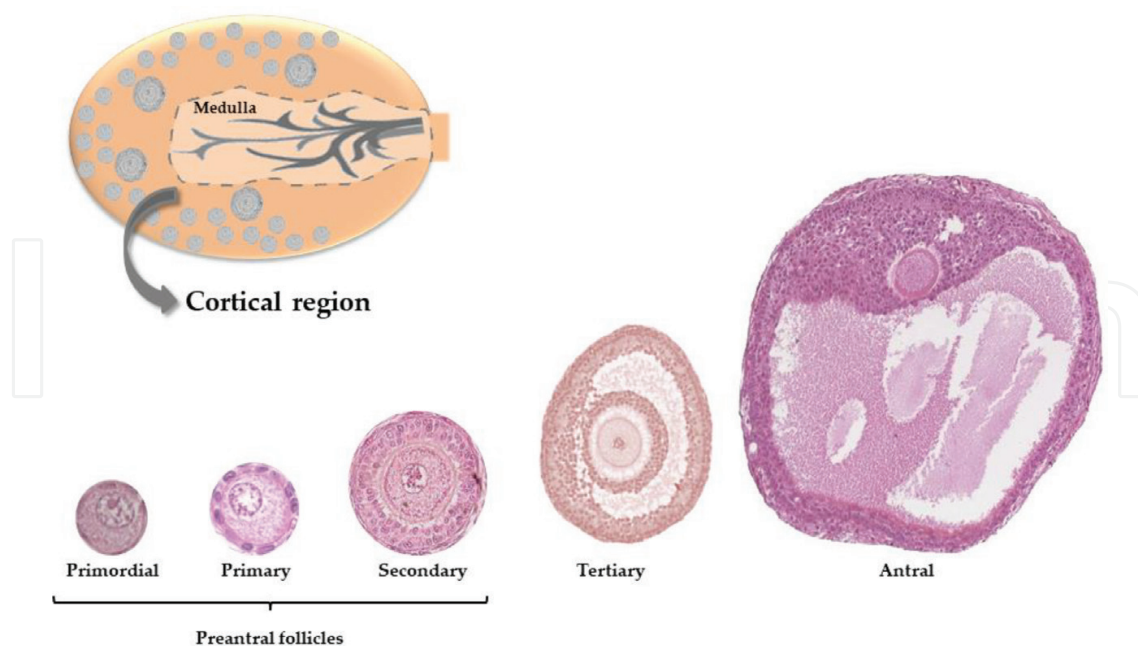


Figure 1. Ovary organization and follicle classification according to developmental stage. Female germ cells are enclosed in follicles that are localized in the cortical region of the ovary, the external layers. The inner layer contains mainly blood vessels and ligaments and is termed the medulla. Once primordial follicles are activated, they start their growth, developing into antral follicles in order to proceed to ovulation. The pictures are not to scale.

preantral follicles do not yet have a zona pellucida or peripheral granules in their cytoplasm, the CPA can easily penetrate. Another advantage of preantral follicle cryopreservation is that they are available in ovaries of all ages, enabling the storage of genetic resources from both young and old, an option not available when cryopreserving fully grown oocytes [7].

Ovarian follicle cryopreservation is now performed in various mammalian species, often with different objectives, which is why researchers need to test and establish appropriate cryopreservation protocols. The goal of this chapter is to summarize some of the recent advances made in the field of ovarian follicle cryopreservation in different mammalian species.

2. Why should we cryopreserve ovarian preantral follicles?

2.1. Indications in women

Cryopreservation of human preantral follicles has proved to be an excellent option to safeguard future fertility. In women, there are three major indications for cryopreserving ovarian tissue containing preantral follicles: malignant diseases or benign conditions threatening fertility or the desire to postpone childbearing or menopause.

Currently, the main reason for cryopreservation of ovarian preantral follicles is to maintain fertility in cancer patients subjected to chemo- and/or radiotherapy. These treatments have different toxic effects on ovarian tissue, including DNA and vascular damage [8], which impair ovarian function [9]. As a result, the follicle pool diminishes, reducing fertility competency and estrogen production and eventually leading to early menopause. The same physiological signs are experienced by healthy menopausal women, whose follicle population declines enough to cease hormone production. In prepubertal patients undergoing gonadotoxic therapy, the storage of germ cells is strongly indicated because oocytes within primordial follicles, which remain in the first meiotic division, are also known to accumulate DNA damage when toxic agents are present [10, 11].

Preantral follicle cryopreservation may be indicated to preserve fertility in patients with nonmalignant conditions that can result in premature ovarian insufficiency. Indeed, certain ovarian pathologies (recurrent ovarian cysts or ovarian torsion), endocrine disorders (galactosemia or Turner syndrome), or diseases requiring chemo- or radiotherapy (autoimmune conditions, aplastic anemia, etc.) can pose a significant threat to fertility [12].

More recently, preantral follicle cryopreservation has also been suggested in the context of healthy women wishing to postpone childbearing. On account of different personal reasons, such as education, career goals, difficulties finding a partner or achieving a stable financial stability, the number of women delaying their first pregnancy has been on the rise worldwide. In most countries belonging to the Organization for Economic Co-operation and Development, the mean age of women giving birth for the first time has increased by 2–4 years in the last 35 years, now standing at 30 years of age or above [13]. As oocyte quality and quantity decline with age, cryopreservation of preantral follicles at a younger age could improve the chances of having a healthy pregnancy and birth.

Finally, an emerging indication for this strategy is to delay the onset of menopause. While life expectancy seldom reached 50 years 100 years ago, meaning most women would die without experiencing menopause, it is now around 80 years in European women, so they live at least 30 of them after menopause [14]. To alleviate symptoms and decrease associated health risks, hormone replacement therapy (HRT) can be prescribed. However, HRT has been linked to a number of health problems, such as stroke, dementia, blood clots, and cancer [15–18]. Preantral follicle cryopreservation could therefore represent a form of “natural” HRT; ovarian tissue could be removed and cryopreserved at a young age, with frozen–thawed fragments subcutaneously transplanted to the patient when she starts presenting with the first signs of menopause [19].

2.2. Indications in other mammalian species

In animals, cryopreservation of preantral follicles can serve different purposes. In the first place, some domestic animal species are important models to develop cryopreservation protocols for human ovarian tissue [20]. Mice, rats, and rabbits are usually chosen because they reach puberty in a short period of time, have a short reproductive cycle, and produce several mature oocytes per cycle. However, research related to the improvement of reproduction capacity in mammalian ovaries also has other purposes today, such as elucidating pathways and mechanisms active in reproductive tissues and generating germ cell cryobanks for endangered species [21].

When cryobanks are created in order to store genetic material from endangered species, assisted reproductive technologies rely on the development of techniques in domestic animal species that show some phylogenetic similarity. For example, dogs [22] and cats [23] have been used as experimental models to develop new techniques to improve available methods for endangered species. It is also important to maintain genetic material from autochthonous breeds, pets, or even production animals. In the latter case, genetically superior animals that show better patterns of production (bovines [24], equines [25], sheep [26], and pigs [27]) are being increasingly studied with a view to enhancing livestock species [28]. This has led to researchers working on the development of cryopreservation protocols specific to different species.

3. Methods for preantral follicle cryopreservation

3.1. Determining the cryopreservation protocol

As with sperm and oocytes, deciding which protocol to use for follicle cryopreservation depends on key factors, such as the type and concentration of CPAs, optimal cooling rates for follicles, the addition of components or extracellular CPAs to improve tissue dehydration, and methods and rates of temperature reduction. Moreover, it is important to bear in mind significant differences in ovarian tissue between mammalian species, which are mainly seen in stromal composition, extracellular matrix (ECM) structure, and follicle morphology and density.

Variations in ECM structure between species are what impacts CPA permeation the most, since it is directly related to cellular connectivity and movement of factors and structures [29].

Perfusion of penetrating CPAs like dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol, and 1,2-propanediol is what causes dehydration of tissue. With low-molecular weights, these compounds are able to penetrate cells and promote an osmotic balance between the compartments that cause cell dehydration, avoiding ice crystal formation. As these compounds are transported from the outer to the inner layers of tissue, dehydration takes place cell by cell, passing through the ECM. Ideal perfusion is reached when cells from the inner tissue areas are filled with CPA [30]. Furthermore, the use of non-permeable CPAs, such as sugars and polymers, is indicated due to their effectiveness in water removal by modifying the osmotic gradient of the system [31]. Sucrose is known to increase cell survival after thawing [27]; effects of trehalose as a membrane-stabilizing agent have also been described [32].

An important point to take into consideration is the CPA concentration; if it is too low, it will not allow adequate cell dehydration. On the contrary, high concentrations cause too much damage due to cell swelling/shrinkage or toxic effects [33]. Any decision about CPA concentrations will essentially depend on the protocol to be used.

Preantral follicles can be cryopreserved by conventional freezing or vitrification. The difference between these two protocols basically hinges on the CPA concentration and cooling rate. Slow-freezing uses low CPA concentrations and seeding, a procedure that promotes the extracellular ice formation, resulting in higher levels of dehydration. In vitrification protocols, ice crystal formation is avoided by an ultra-fast temperature reduction associated with high CPA concentrations, which may in turn lead to cell toxicity. As an alternative, liquidus tracking systems have been developed, aiming to reduce tissue/follicle damage from these concentrations. Stepped vitrification may be performed and the cell response to CPA toxicity may be attenuated, since the sample is only subjected to high concentrations of CPA when low temperatures are experienced in the local environment, hence lowering cell metabolism and activity [34, 35]. Some examples of cryopreservation solutions and equilibration curves applied before storage are shown in **Table 1**.

Follicles can be cryopreserved inside ovarian tissue or after isolation from it. Both techniques have been applied in several animal species and have shown advantages and disadvantages.

3.2. Ovarian tissue cryopreservation

The heterogeneous cell composition of ovarian tissue presents different challenges in terms of CPA perfusion and cooling rates to establish an optimal cryopreservation protocol. Not only do cells deserve our attention, but also the extracellular components, since the ECM and basement membranes must be maintained in order to provide an adequate structure when the tissue is warmed and transplanted [33]. As the oocyte is the target cell, most protocols for ovarian tissue cryopreservation are derived from those applied to mature oocytes.

Ovarian tissue can be cryopreserved in its entirety or cut into halves or small pieces. Various ovarian tissue cryopreservation protocols for different species are shown in **Table 2**. In sheep,

Species	Approach	Medium	Non-permeable cryoprotectant	Proteins	Equilibration curve	Reference
Human	Slow-freezing	MEM	Not used	HSA	0 °C → -8 °C (-2°C/min) -8°C → -40°C (-0.3°C/min) → -196°C	Amorim <i>et al.</i> [36]
	Slow-freezing	PBS	Sucrose	Not used	1°C → -9°C (2°C/min) → -40°C (-0.3°C/min) → -140°C (-10°C/min) → -196°C	Schmidt <i>et al.</i> [37]
	Vitrification	TCM199	PVP and sucrose	Not used	Direct immersion in LN	Suzuki <i>et al.</i> [38]
Baboon	Vitrification	MEM	PVP and sucrose	HSA	Direct immersion in LN	Amorim <i>et al.</i> [39]
Cow	Vitrification	PBS	Not used	Not used	RT→0°C	Corral <i>et al.</i> [35]
					0°C → -4°C (-3°C/min) → -8°C (-3°C/min) → -40°C (-3°C/min) → -150°C (-20°C/min) → -196°C	
Sheep	Slow-freezing	L-15	Not used	Calf serum	4°C → -7°C (-2°C/min) → -40°C (-0.3°C/min) → -140°C (-10°C/min) → -196°C	Gosden <i>et al.</i> [4]

LN, liquid nitrogen; HSA, human serum albumin; L-15, Leibovitz-15; MEM, minimum essential medium; PBS, phosphate buffered saline; PVP, polyvinylpyrrolidone; RT, room temperature.

Table 1. Examples of some cryopreservation solution contents applied for ovarian tissue slow-freezing or vitrification.

for example, cryopreservation of whole ovaries was successfully achieved [40]. It involved special CPA perfusion techniques because the structure is much larger. Such techniques can include immersion of the ovary in a CPA solution and also perfusion of CPAs with needles and clamps in order to inject the solution through the ovarian artery [41].

Cryopreservation of small tissue pieces is more commonly performed, since thinner layers allow smoother CPA permeation. As preantral follicles are usually present in the outer layer of the ovary (cortex), this area is chosen when a biopsy is taken for follicle cryopreservation. In addition, the thinner the ovarian piece, the lesser it will experience damages due to oxidative stress and reactive oxygen species (ROS) formation until its nutrition and oxygenation are reestablished, especially because the freeze–thaw process can make cells more sensitive to ROS effects [42].

The mechanism of passive carriage of CPAs throughout cells also depends on the activity of transmembrane proteins like aquaporins [43]. In oocytes, it is known that CPAs, such as DMSO and EG, increase aquaporins expression after cryopreservation [44]. These permeating CPAs are frequently used for ovarian tissue cryopreservation. So far, DMSO has proved more

Species	Ovarian strip size	Cryoprotectant	Cryopreservation technique	Procedure post-thawing	Results	Reference
Human	2 × 2 mm	1.5 mmol/l DMSO	SF	Autotransplantation	Live birth	Donnez <i>et al.</i> [57]
Mouse	¼ ovary	15% glycerol	SF	Allotransplantation	Live birth	Parrot [58]
	Half ovary	Commercial kit	Vitrification	Allotransplantation	Live birth	Okamoto <i>et al.</i> [50]
Rat	3 × 3 × 1 mm	1.5 M DMSO	SF	Autotransplantation	Follicle activation	Celik <i>et al.</i> [59]
	1 mm ³	Commercial kit	Vitrification	Autotransplantation	Reestablishment of ovarian function	Wietcovsky <i>et al.</i> [60]
Cat	2 × 2 × 1 mm	1.5 M EG	SF	Xenotransplantation	Follicle growth to antral stage	Bosch <i>et al.</i> [61]
Dog	3 × 3 × 1 mm	1.5 M DMSO	SF	Morphology and follicle viability	67.5% of viable follicles	Lopes <i>et al.</i> [45]
Deer	2 × 2 × 0.5 mm	20% EG + 20% DMSO	Vitrification	IVC	Viability of preantral follicles evaluated by IHC	Gastal <i>et al.</i> [53]
Cow	5 × 5 × 0.5 mm	15% DMSO +15% EG	Vitrification	Xenotransplantation	Follicle growth to antral stage	Bao <i>et al.</i> [62]
Sheep	Whole ovary	1.5 mol/l DMSO	SF	Autotransplantation	Live birth	Campbell <i>et al.</i> [40]
	1 mm thick	1.5 M DMSO	SF	Autotransplantation	Live birth	Gosden <i>et al.</i> [4]
Mare	3 × 3 × 0.5 mm	3 M EG for vitrification; 1.5 M DMSO for SF	Vitrification and SF	IVC	Cell viability	Gastal <i>et al.</i> [25]

DMSO, dimethyl sulfoxide; EG, ethylene glycol; SF, slow-freezing; IVC, *in vitro* culture.

Table 2. Tissue size, main CPA concentrations, cryopreservation techniques, and results from published protocols for ovarian tissue cryopreservation in a variety of species.

efficient in some species, including bitches [45], goats [46], mares [25], and sheep [47] while EG is usually used in association with DMSO [48, 49].

Regarding cryopreservation technique, some authors extol the advantages and effectiveness of ovarian tissue vitrification [38, 50], but slow-freezing remains the method of choice for humans. Interestingly, the protocol described by Gosden *et al.* back in 1994 [4] is still routinely used for cryopreservation of human ovarian tissue, with some small modifications [51]. This method involves a curve that usually begins with a temperature reduction of 2°C/min to -7°C, followed by seeding; then another reduction to -40°C at 0.3°C/min, and finally plunging into liquid nitrogen (**Figure 2**) [19]. Although slow-freezing is the first-line approach in certain species, vitrification is the best alternative when compared to others [52–54].

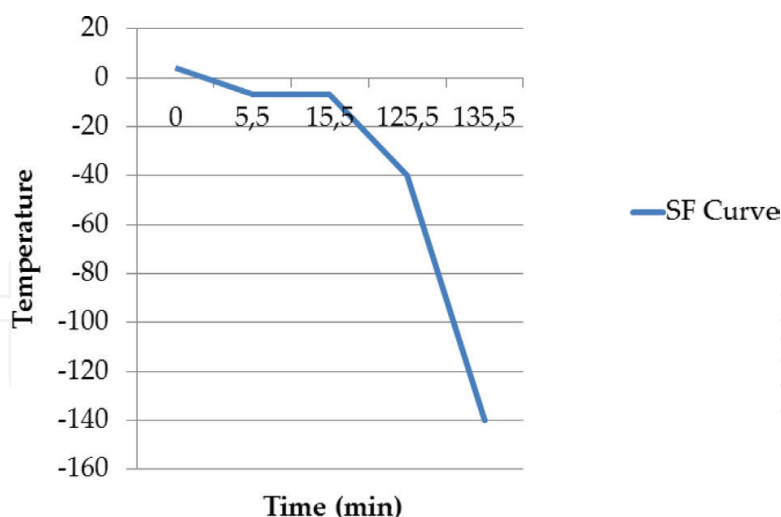


Figure 2. A schematic demonstration of the cooling rate applied by Gosden *et al.* [4] for slow-freezing of sheep ovarian tissue. Usually, cooling rates are performed in an automatic freezer, useful for cooling samples up to -140°C . SL, slow-freezing.

After thawing, ovarian tissue can be used for transplantation, *in vitro* culture, or follicle isolation. Nowadays, transplantation techniques are widespread, and more than 130 human live births have been documented worldwide following ovarian tissue cryopreservation and transplantation [55]. Such success rates have led to a greater visibility of this procedure in hospitals around the world. Indeed, in some countries like Israel, this strategy is no longer considered experimental [56].

3.3. Isolated follicles cryopreservation

While cryopreservation of isolated follicles is less commonly described in the study, it has some key advantages. If tissue cryopreservation may suffer impairment due to difficult CPA perfusion, this issue is somewhat reduced in case of isolated structures. Preantral follicles are small (usually less than $150\text{ }\mu\text{m}$ in diameter), so CPA perfusion is facilitated and optimal concentrations are easier to gauge in oocytes. Moreover, CPA types and concentrations as well as cryopreservation procedures can be precisely tailored to preantral follicles, taking into consideration their permeability parameters [63–65]. Another advantage of using isolated follicles is more specific to humans; in some types of cancer, there is a risk of malignant cells being present in the ovarian tissue, so transplantation is not recommended. The use of isolated follicles instead of vascularized ovarian tissue avoids the risk of reintroducing the disease [66], since their basal membrane prevents them from coming into contact with malignant cells that may be present in the tissue [67]. Another point worth mentioning is the considerable follicular loss that occurs due to ischemia–reperfusion after transplantation of ovarian tissue, which could be avoided by grafting isolated follicles [68].

On the other hand, there are limitations, like follicle dissociation from the surrounding ovarian tissue. To isolate preantral follicles, we can use mechanical [69] or enzymatic [70] means, or an association of both, depending on the origin of the ovarian tissue [71]. The mechanical

dissociation of the follicles is based on its fragmentation; cutting the cortex into small pieces with scissors or even with surgical blades are some options; in addition, the use of a tissue chopper has been widely described [71–73]. Most studies on human follicle isolation use liberase and/or collagenase for enzymatic digestion [70, 71, 74, 75]. This step is crucial, and care must be taken to avoid or mitigate the chances of follicle damage during these procedures. Choosing the right enzyme and an adequate concentration are vital and must be well thought out, since differences in the fibrous nature of the ovary [76] and basal membrane composition of various animal species [77] require specific isolation protocols for the different types of ovary. Indeed, even in the same species, ovary composition changes with age, so follicle isolation may well need an individually tailored approach [71].

The first successful cryopreservation of isolated follicles was achieved in mice, when Carrol *et al.* obtained offspring after follicle isolation, cryopreservation, *in vitro* culture, maturation, fertilization, and finally embryo transfer [78]. More recently, cryopreservation of isolated follicles has been performed in a much greater number of animal species (**Table 3**).

The routine procedure for cryopreserving isolated follicles is similar to that used for oocytes and isolated cells in general. After isolation, they are submerged in CPA solution and placed in a plastic straw for freezing [88].

Unlike ovarian tissue, isolated follicles cannot be immediately transplanted after thawing; they first need to be encapsulated in a matrix, made of fibrin [89], alginate [90], collagen [91], and/or other materials [92] in order to maintain their 3D structure. This also allows better handling of the follicles, facilitating cryopreservation, *in vitro* culture, and transplantation. Isolated follicles can also be encapsulated in a matrix prior to cryopreservation. To date, only an alginate matrix has been used to cryopreserve follicles after isolation [80].

Species	Cryoprotectant and its final concentration	Method	Reference
Human	1.4 M DMSO	SF in sodium alginate matrix	Camboni <i>et al.</i> [79]
	40% EG	Vitrification	Bian <i>et al.</i> [80]
Goat	1 M EG + 0.5 M sucrose	SF	Santos <i>et al.</i> [81]
Sheep	2.6 M acetamide, 2.62 M DMSO, 1.31 M 1,2 propanediol and 0.0075 M polyethylene glycol	Vitrification	Lunardi <i>et al.</i> [82]
Cat	1.5 M DMSO or 1.5 M EG	SF	Jewgenow <i>et al.</i> [83]
Rat	35% EG + 0.5 M sucrose	Vitrification	Xing <i>et al.</i> [84]
Mouse	6 M EG	Vitrification	Desai <i>et al.</i> [85]
	15% EG + 15% DMSO	Vitrification	Ganji <i>et al.</i> [86]
Monkey	8.83% EG + 35 mg/ml sucrose	SF	Barret <i>et al.</i> [87]

DMSO, dimethyl sulfoxide; EG, ethylene glycol; SF, slow-freezing.

Table 3. CPAs, their concentrations, and cryopreservation methods for isolated ovarian preantral follicles.

4. Warming rate and CPA removal

The impact of the warming rate is another important point to be taken into account. Due to the risk of ice formation during warming, fast protocols involving immediate plunging of the cryovials into a water bath at 30–40°C, are more frequently applied [45, 54, 93]. Indeed, cooling and warming rates interact, and both are keys to achieving a favorable outcome. Akhtar *et al.* [94] compared different cooling and warming rates for cryopreservation of lymphoma cells and reported that the best results were obtained with a conventional slow-cooling (1°C/min) and fast-warming (200°C/min) protocol [94]. In vitrification protocols, the warming rate is of much greater importance, particularly when high CPA concentrations were not adequately achieved. In this case, there is a risk of ice crystal formation during rewarming that may be avoided with very rapid warming rates [95]. When permeating CPAs are removed, an osmotic imbalance usually occurs; there is an uptake of water causing the cells to swell, increasing their natural volume. As the CPA is eliminated, together with the water, the cells start to shrink again, aiming to recover their osmotic equilibrium. A physiologically normal volume is reestablished only when no natural solutes are able to leave or enter the cells [96]. In order to define the optimal CPA concentration that can induce sufficient cell dehydration and prevent damages caused by cell swelling/shrinking during CPA removal, further tests must investigate how much variation each cell type can tolerate in terms of its volume [65]. The use of non-permeable CPAs like sugars and polymers is known to help in the removal of their permeating counterparts and have a protective effect on cell membranes [32].

5. Conclusion and final considerations

In addition to CPA effectiveness for cell preservation, it is vital that we investigate possible long-term toxic effects on cells in frozen tissue, or, indeed, on the host after transplantation. Long-term studies on the impact of CPAs on mature oocytes and embryos resulting from these cryopreserved follicles should also be carefully analyzed. Epigenetic alterations to the DNA of cells subjected to CPAs may emerge. For instance, DMSO is known to produce modifications to DNA methylation in embryos [97]. Despite a limited number of studies on ovarian tissue, data on other tissues provide valuable information. Even low DMSO concentrations in blood can cause damage to the central nervous system during development [98], and teratogenic effects have been described, as having alterations to lipid metabolism [99].

In summary, the different options available to cryopreserve ovarian preantral follicles have both benefits and limitations, some of which are cited in **Table 4**. Attempts made so far with single-cell system protocols for tissue cryopreservation have resulted in failure, showing that being able to adapt is fundamental. Although existing data show that ovarian tissue cryopreservation and transplantation is feasible and effective, follicle loss is still an obstacle to be surmounted. Thus, the protocol of choice will depend on a variety of factors, including the goal of follicle cryopreservation, its purpose after thawing, and the availability of laboratory equipment.

It is undeniable that preantral follicle cryopreservation can help patients face the challenges of chemotherapy, improving their chances of fertility restoration once they are cured. Moreover,

Feature	Ovarian tissue	Isolated follicles
Transplantation safety in terms of disease reintroduction	Low	High
Transplantation procedure	Facilitated	Difficult
CPA penetration	Difficult	Facilitated
Follicle loss (transplantation)	High	Low (controlled)
CPA, cryoprotective agent.		

Table 4. Pros and cons of both strategies used to cryopreserve preantral follicles.

this may be the only strategy available now to preserve female germ cells of highly endangered animal species. It is nevertheless important to stress that while currently implemented cryopreservation procedures have yielded successful results, there is still room for improvement. Studies should be performed to enhance outcomes and facilitate the creation of cryobanks in medical centers and animal facilities worldwide.

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