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Applications and Challenges

Edited by Marian Quain

Cryopreservation - Applications and Challenges

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Biomedical Engineering

Volume 18

Aims and Scope of the Series

Biomedical Engineering is one of the fastest-growing interdisciplinary branches of science and industry. The combination of electronics and computer science with biology and medicine has improved patient diagnosis, reduced rehabilitation time, and helped to facilitate a better quality of life. Nowadays, all medical imaging devices, medical instruments, or new laboratory techniques result from the cooperation of specialists in various fields. The series of Biomedical Engineering books covers such areas of knowledge as chemistry, physics, electronics, medicine, and biology. This series is intended for doctors, engineers, and scientists involved in biomedical engineering or those wanting to start working in this field.

Meet the Series Editor

Robert Koprowski, MD (1997), Ph.D. (2003), Habilitation (2015), is an employee of the University of Silesia, Poland, Institute of Computer Science, Department of Biomedical Computer Systems. For 20 years, he has studied the analysis and processing of biomedical images, emphasizing the full automation of measurement for a large inter-individual variability of patients. Dr. Koprowski has authored more than a hundred research papers with dozens in impact factor (IF) journals and has authored or co-authored six books. Additionally, he is the author of several national and international patents in the field of biomedical devices and imaging. Since 2011, he has been a reviewer of grants and projects (including EU projects) in biomedical engineering.

Meet the Volume Editor

Marian D. Quain is a chief research scientist with the CSIR-Crops Research Institute, Ghana. She is a biotechnologist whose research focuses on the utilization of tissue culture techniques for the production of clean planting materials, germplasm conservation, and the use of molecular tools for crop diversity, disease diagnostics, and molecular marker-assisted selection breeding using transcriptomics and proteomics, as well as application of recombinant gene technology for crop improvement. During 2011–2013, she was a visiting research fellow at Leeds University, UK. She is a 2017 Cochran fellow and a fellow of the Africa Science Leadership Program. In 2018, she won the American Society for Plant Biologists Award for Excellence in Education. In 2022 she was celebrated as Biotechnology Hero by the African Agricultural Technology Foundation.

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Preface

Genetic resources represent the genetic storehouse of information, which needs to be explored, evaluated, cataloged, and conserved so that no genotype is lost from the treasury of genome diversity. Plant genetic resources (PGRs) are essential components of agro-biodiversity and are defined as the genetic material of plants having value as a resource for present and future generations [1]. With no limitation to plants, animal genetic resources (AnGRs) can be referred to as genetic diversity in domesticated animal species with economic or other socio-cultural values and found among species, among animal breeds within the species, and in cryopreserved material in the form of embryos and semen [2].

Posterity is essential and it is critical that all available methods including cryo-preservation applications are explored to maintain the available scope of diversity. Cryopreservation, which is the cooling of cells to sub-zero temperatures, at which all metabolic systems are preserved indefinitely, may be an illusion due to the associated difficulties in revitalizing cells after cooling. However, when successful, this method holds the future for cell preservation.

Major parameters that require attention for a successful cryopreservation procedure include the method of cooling, type of cryoprotectant, recovery and revitalization after preservation, and type of cells or tissues being preserved (e.g., plant parts, germlines, etc.). Cryoprotection is the process during which cells to be cryopreserved are treated with substances that will protect the tissues from freezing damage. Freezing damage occurs when inter- and intracellular fluids form ice crystals that perforate membranes and organelles thus damaging their viability. The formation of glass in cells instead of crystals prevents this damage, and this can be facilitated using cryoprotective agents (CPAs). The formation of glass instead of ice crystals is referred to as vitrification. CPAs are thought to inhibit ice formation by interacting with hydrogen bonding among water molecules [3], resulting in the production of nonspecific toxic effects [4].

The most known CPAs are dimethyl sulfoxide (DMSO), glycerol, ethylene glycol (EG), 2-Methyl-2,4-pentanedol (MPD), propylene glycol, sucrose, and trehalose. The CPAs can be categorized as either membrane permeating, such as glycerol, ethylene glycol, and DMSO, which can freely diffuse the membrane, and non-membrane per-meating, such as sugars, which cannot permeate the cell membrane. Although useful, CPAs may be toxic to cells, causing biological material to compromise their genetic makeup, metabolic systems, and functionality. Factors that must be controlled when using CPAs include the concentration of the cryoprotectant, duration of exposure, and combination of two or more cryoprotectants.

This book is a review of research outputs, proposals, and challenges associated with using cryopreservation techniques to conserve germplasm for posterity. It considers

various types of plant parts and germlines used for conservation. Successful preparation of cells and tissues prior to cooling is critical to ensure restoration after cooling as is the appropriate use of cryoprotectants.

This book includes two sections. Section 1, "Gametes and Embryos Cryopreservation", includes 5 chapters.

Chapter 1, "Cryopreservation Studies in Aquaculture from Past to Present: Scientific Techniques and Quality Controls for Commercial Applications", sheds light on the cryopreservation of sperms of various fish species, namely, carp, sturgeon, eel, salmonid, and catfish, some of which are endangered species. The chapter reviews the conservation of germ cells and embryos. It also discusses contamination that may be associated with fish gamete cryopreservation and the thawing process. It presents a thorough review of successful procedures, disease transmission via gametes to the embryo, elimination of non-cellular disease agents from gametes, antibiotics, sperm washing and cell separating methods, and disinfection of gametes and embryos.

Chapter 2, "The Current Status of Semen and Oocytes Cryopreservation", reviews challenges associated with the cryopreservation of gametes with specific references to oocytes and sperms. Adequate reference is made to the effect of these challenges on in vitro fertilisation (IVF). It also discusses the use of cryoprotectants, antioxidants, and various freezing methods.

Chapter 3, "Female Fertility Preservation: Different Interventions and Procedures", discusses cryoprotectants, particularly permeable cryoprotective agents (CPAs) such as glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PG). It also discusses non-permeable cryoprotectants as well as cooling methods such as controlled slow-rate freezing and vitrification. The chapter discusses the use of permeable and non-permeable CPAs, their mode of action, and their toxic effects. It is suggested that a mixture of cryoprotectants is less toxic and more effective for successful cryoprotection. The non-permeable CPAs are presented as remaining in extracellular space and having the ability to reduce the formation of extracellular ice formation. Ovarian tissue cryopreservation is reported as an evolving technique that requires more standardised applications.

Subsequently, Chapter 4, "Ovarian Tissue Cryopreservation Guidelines", highlights ten systems that could serve as guidelines for successful cryopreservation and discusses their associated challenges.

Chapter 5, "Scaling up Cryopreservation from Cell Suspensions to Tissues: Challenges and Successes", addresses critical topics associated with CPAs, the dimension and complexity of cells, ice formation, and cooling rate. A thorough review is provided on cryopreservation of larger structures like the ovaries, thymus, and biopsies. The chapter also presents ways to overcome practical challenges associated with CPA loading and unloading, diffusion of heat and intracellular water, ice nucleation and direct ice damage, and control of ice structure.

Section 2 includes two chapters that focus on the prospects of cryopreservation as applied in blood and plants. Chapter 6, "Impact of Different Cooling Methods on the Stability of Peripheral Blood Mononuclear Cells (PBMCs)", highlights cooling

methods and makes recommendations based on research activities conducted by the authors. Three cooling methods are discussed: -1° C/min cooling rate that requires only isopropyl alcohol, a cooling rate of -1° C/min solely, and a user-predefined programmable controlled rate of freezing. The method using isopropyl alcohol is not recommended, whereas the two other cooling methods are recommended for cryopreservation of peripheral blood mononuclear cells (PBMCs).

As the world grapples with the threats of climate change and food insecurity, the fate of clonally propagated crops is crucial and cryopreservation techniques must be considered.

Chapter 7, "Plant Cryopreservation Importance, Approaches and Future Trends", reviews various affordable cooling systems with attention to the removal of freezable water and proposes a way forward that includes the development of required technical expertise.

This book provides a comprehensive overview of cryopreservation in cell therapies, tissue-engineered constructs, and other larger tissues.

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Section 1

Gametes and Embryos Cryopreservation

Chapter 1

Cryopreservation Studies in Aquaculture from Past to Present: Scientific Techniques and Quality Controls for Commercial Applications

Aygül Ekici, Güneş Yamaner and Menekşe Didem Demircan

Abstract

In this section, cryopreservation of fish genetic resources, which is one of the important applications to ensure the sustainability of genetic resources of freshwater fish species, is discussed. At the same time, information is provided about the possible sources of contamination that may be encountered during cryopreservation applications. In this context, the results of sperm, egg, and embryo cryopreservation studies of fish and their success and failure in applications were evaluated in addition to the process from past to present. Information is given about the contamination that may develop depending on the applications in the process of cryopreservation and dissolving processes, as well as the studies carried out to eliminate extracellular disease agents. In the section, in addition to the evaluation of the results of scientific studies, commercial companies that commercially carry out gamete cryopreservation applications are also included. The contamination that may develop depending on the applications in the process of cryopreservation and thawing processes, as well as the studies carried out to eliminate extracellular disease agents are mentioned.

Keywords: sperm, egg, germ cell, storage, contamination

1. Introduction

Developing gamete conservation programs will be invaluable in future commercial fish reproductive studies. Gamete conservation is an important tool for fish reproduction and is very important for aquaculture. It may be widely used in breeding laboratories. The growing interest in improving technology has led to an increase in the number of studies on this subject. In particular, sperm has an important place in cryopreservation studies and is still performed both in laboratory studies and in culture applications [1, 2]. Although the freezing of male sperm dates back to the 1600s, the successful method of artificial insemination at the end of the 1950s, with the need for long-term storage of sperm. In 1953, once the method of freezing sperm was successful with the

herring (*Clupea harengus*) that achieved approximately 80% cellular motility upon thawing [3]. After that, the growing interest in improving preservation technology has increased the number of studies on sperm cryopreservation. Therefore, cryopreserved sperm can now be used in routine fish reproduction and aquaculture practices [4].

Before achieving standardization in the sperm cryopreservation procedure, aquaculture conditions, broodstock management, standardization of the feeding regime, and the production of disease-free lineages are essential for the success of standardization. In the next stage, providing appropriate biosecurity conditions in the laboratory where sperm cryopreservation process will be performed will be an important step that increases the success and brings it closer to standardization. Once a standard procedure has been established, for example, the kits used in DNA and RNA isolation, standardization can be achieved by creating fish species-specific sperm cryopreservation kits.

Although sperm cryopreservation studies have focused on Cyprinid, Sturgeon Salmonids, and Catfish species that are intensively cultured, there are many cases of successful freezing studies with other various fish species [5–7]. Since a review on cryopreservation of fish sperm was carried out before [8], the work in this present section will be focused on more recent improvements in fish (carp, sturgeon, eel, Salmonid, and catfish) sperm cryopreservation with the extender (+cryoprotectant)/additive material and freezing/thawing procedure, mostly.

2. Cryopreservation of freshwater fish sperm

Populations of aquatic species are threatened by anthropogenic influences, overfishing and poaching, destruction of spawning habitats, as well as an increase in water temperature caused by climate change. Today, fish farms carry out production activities by preserving live fish. However; in addition, because of problem with water resources, gametes, genetic factors, disease-related factors, operational problems, system failure, environmental problems, and the survival chances of these creatures become difficult.

High volume and high-quality sperm quantity must be obtained for the commercialization of sperm cryopreservation applications. The successful use of cryopreserved sperm in gene banks in fertilization studies requires that the preserved material is of an acceptable quality/viability and quantity [9]. A company based in Norway provides the aquaculture industry with cryopreserved sperm of 16 fish species, including 8 species belonging to the Salmonidae family [10]. In addition, sperm preservation, maturation, and cryopreservation solutions specific to aquatic species are commercially available on the market [11]. There are also some solutions developed by various commercial companies for use in tissue and cell freezing.

In sperm cryopreservation studies, the goal is to achieve the highest fertilization rate with frozen sperm. However, the failure to achieve similar results in repeated experiments in sperm cryopreservation studies, using of this application in aquaculture studies is limited [12]. Standardization is the biggest problem in sperm cryopreservation, but it will be useful to understand the molecular mechanisms by using new-generation technologies to ensure standardization.

2.1 Carp sperm cryopreservation

The common carp (*Cyprinus carpio*) is one of the largest farmed freshwater fish in the world, with its production reaching 4363.3 thousand tons in 2020 [13]. In addition

to being of commercial interest, it is also a model organism within Teleostei, and is one of the first cultured fish [14]. The sperm cryopreservation of carp was developed several decades ago, and cryopreservation of sperm has often been investigated in carp due to its economic (commercial) and recreational value. However, Carp have undergone selective breeding, resulting in different strains. Cryopreservation can be used to secure sperm from the desired common carp strains in sperm banks and for sperm transport [8, 15]. Therefore, there is a growing interest in methodological and practical innovation in the cryopreservation of carp sperm. Even though the protocols for cryopreservation of carp sperm have been developed [16–19], reduction in sperm viability and motility is still observed in cryopreserved sperm. Therefore, before the cryopreservation of sperm, a thorough evaluation of different extender solutions with cryoprotectant, additives material (cyclodextrin, ext.,) and cooling or thawing rates are essential to develop optimum cryopreservation protocols for the carp.

An extender is a chemical compound used in sperm cryopreservation studies and includes inorganic chemicals. The extender has to be similar to blood and sperm seminal plasma in terms of the inorganic chemicals it contains in order for sperm cells to maintain their viability in vitro preservation. Also, the extender functions as nutrients, pH regulators, and seminal plasm osmotic pressure. Therefore, the extender used to freeze the sperm of freshwater fish should be specific to the species. Since the sperm activation mechanism of each species is different, the extenders used vary. Cryoprotectants are added for protection and stored in a cryogen that can produce very low temperature due to its varied state (e.g., liquid nitrogen at −196°C) [8].

A number of efficient cryo-medium context has been developed for carp sperm. One of the most important steps in the successful cryopreservation of carp sperm is selecting the cryoprotectant in the extender during the process. Various cryoprotectants such as glycerol, DMSO, methanol, and DMA have been used as cryoprotectants for fish sperm. Some studies show that DMSO works best as cryoprotectant in carp sperm compared to glycerol and methanol. In another study conducted with carp, it was emphasized that the use of DMSO at a rate of 5–20% was effective in the sperm of carp [16–19]. Another cryoprotectant used to freeze carp sperm is the yolk of an egg. It has been reported that duck egg yolk, which is used as an extracellular cryoprotectant in cryopreservation sperm of carp, increases motility after thawing and the fertilization rate compared to chicken egg yolk [20].

In addition to the studies investigating the effectiveness and success of cryoprotectants in the freezing process of carp sperm, there are studies that revealing the oxidative stress caused by dilution with cryoprotectants during/before the cryopreservation process. Seminal plasma protects sperm cells against oxidative stress. Dilution of sperm cells during cryopreservation reduces the seminal plasma content, which makes sperm cells more sensitive to oxidative stress [21]. Therefore studies have focused on reducing the effect of oxidative stress in the cell. The fact that amino acids have antioxidant properties and are present in high concentrations in seminal plasma has made amino acids an important component in sperm cryopreservation studies [21–24]. It has been shown that the use of L-cysteine in the process of sperm freezing in carp has a positive effect on motility and viability of sperm cells [25]. It has been stated that the use of Cysteine at 20 mM in the freezing medium of carp sperm process makes a significant difference in motility and motility time [26]. Another additive used is cholesterol-laden cyclodextrin (CLC) to reduce cell damage during the freezing process. In the freezing of carp sperm, the use of 1.5 mg of CLC in the extender was found to have the best cryoprotective effect in maintaining sperm motility, duration, and viability of sperm cells [27].

In addition to the cryoprotectant studies mentioned above, one of the factors affecting the freezing process and is the density of the sperm before freezing. It has been reported that sperm concentration is often ignored before freezing, and the sperm and extender are diluted in a certain volume-to-volume ratio [28, 29]. On the other hand, it has been shown that optimization of sperm concentration plays a crucial role in a number of aquaculture species, in particular pikeperch (*Sander lucioperca*) [30], European perch (*Perca fluviatilis*) [9], and Salmonid [31]. However, it was observed that there was no significant difference between sperm samples' motility and velocity after thawing when different sperm concentrations were specially adjusted before freezing in carp sperm [32].

Besides studies on the effect of chemical and non-chemical components used in freezing sperm, studies on the freezing process are varied. In adding to studies on the freezing procedure with liquid nitrogen and liquid nitrogen vapor used for a long time, studies are also performed with programmable freezing devices. In studies using liquid nitrogen vapor and liquid nitrogen, the heights of straws to liquid nitrogen vapor and straws volumes were studied. In Ref. [33], tested different freezing rates by modifying the height (2–6 cm) above the surface of liquid nitrogen, where the straws were placed. The highest fertilization was observed when samples were frozen at 2 cm above the level of liquid nitrogen and 10 min freezing time (74 ± 7%).

2.2 Sturgeon sperm cryopreservation

Sturgeons are the oldest freshwater fishes, having evolved around 200–250 million years ago [34]. The high economic value of sturgeon, mainly because of their caviar, the failure to manage the caviar trade, and unsustainable fishing (in the seas and river), along with serious habitat fragmentation have led to a significant decline of wild sturgeon populations [35, 36]. Due to this decrease in natural stocks, all sturgeon species have been included in the list of endangered species under CITES (Convention on International Trade in Endangered Species) since 1997 for population restoration; however, thanks to aquaculture, the sturgeon still maintains its place in the valuable product category in fish markets worldwide [37]. Sturgeon culture is developing and increasing in order to meet the need for products obtained from sturgeon and, in addition, support natural stocks. Another method of protection of natural stocks, as already described, is the cryopreservation of gametes or embryos. Especially fish sperm cryobanking is considered a potentially powerful tool in aquaculture for endangered species [38].

Gamete cryoperservation in sturgeon has been given more importance because the species is one of the extinct species. And in sturgeon species, especially sperm freezing, biological, mechanical, and biochemical factors affecting sperm cryopreservation have been studied extensively in order to increase post-thawing motility. Since there is literature [8] on this subject before, current research topics of recent years are given in this section.

The most commonly used cryoprotectant for cryopreservation sperm of sturgeon is methanol. In various species of sturgeon, the lowest fertilization rate obtained using this cryoprotectant 6% (Siberian sturgeon, *Acipenser baeri*); the highest was reported as 40% (Shortnose sturgeon, *A. brevirostrum*) [39–41]. In Russian sturgeon (*A. gueldenstaedtii*), the use of 10% methanol in sperm cryopreservation causes a decrease in acrosin activity and an increase in DNA damage; however, compared to the solution in which methanol was not used, it was reported that this cryoprotectant protects sperm cells during cryopreservation process [42]. In sperm cryopreservation

in sturgeon, in addition to intracellular cryoprotectant substances, there are studies in which cryoproctants such as sugars, egg yolk, and vegetable oil, which are called extracellular, are tested [43]. In the study conducted with Persian (*A. persicus*) and Beluga (*Huso huso*) sturgeon sperm; the addition of different disaccharides such as maltose, trehalose, lactose, and lactulose along with 9% methanol solution has been reported to have an effect on motile sperm cells, except for lactulose. The authors also reported that the cryopreserved sperm using each of the four disaccharides could be stored for at least 30 min without losing sperm motility [44]. In addition to the studies reporting that the protective effect of disaccharides during sperm freezing is due to the high molecular weight they have [44], there are studies reporting that this protective effect is entirely due to the chemical structure of the disaccharides [45, 46]. When the use of sucrose or trehalose alone and in combination with different concentrations was tested for cryopreservation of Dabry's sturgeon (*Acipenser dabryanus*) sperm, low concentration sucrose plus trehalose ($S_{15}T_{15}$) solution was reported to be the optimal solution. Also, it was reported that mixing of the extender with sucrose, lactose, or trehalose alone or with pairwise mixtures revealed that a mixture of lactose and trehalose (L15T15) gave the best results for both Chinese sturgeon (*A. sinensis*) and Dabry's sturgeon [47].

Along with cryoprotectants that protect the viability of sperm cells, various dilution agents that provide the dilution of the sperm and reduce the cell damage seen in the freezing process are also used. Another substance added in cryomedium in the freezing of sturgeon sperm is antioxidant substances. These substances, which reduce oxidative stress in the sperm cell during the freezing process and therefore increase sperm quality after thawing, are ascorbic acid, catalase, glutathione, and cysteine [48]. Although the protective effect of using catalase (25 U/mL), glutathione (0.25–0.5 mg/mL), and ascorbic acid (0.5 mg/mL) in freezing the sperm of three species of sturgeon (*A. dabryanus*, *A. sinensis*, and *Acipenser baerii*) on sperm cells have been mentioned; it has been reported that the three antioxidants should not be used together [49].

In order to protect sperm cells from cryodamage in sperm cryopreservation in many fish species, including sturgeon, it is recommended to use various proteins, enzymatic or non-enzymatic antioxidants, and antifreeze proteins in the cryopreservation procedure [50]. However, the effect of antifreezing protein on sperm cells during freezing in sterlet sturgeon was examined and it was found that a significant decrease in motility rate and velocity of curvilinear (VCL) was observed in cryopreserved spermatozoa with/without supplementation of 10 g AFPI compared to fresh spermatozoa. And also the results showed that in partial changes in the ultrastructural compartments, weakening of the midpiece and rupture of the plasma membrane of the flagellum were seen. The author believes that this damage is not due to oxidative stress that can occur in cryopreserved sperm; expressed that there is physical damage that occurs during the formation of ice crystals during freezing process [51].

It is a known fact that after cryopreservation of the sperm, the motility in the sperm cells decreases and if these sperm samples are used in the fertilization study, a low fertilization rate will be obtained [52]. All the studies carried out so far have been aimed at increasing sperm viability/motility, that is, the fertility of sperm, after thawing. However, in the sperm cryopreservation study using methanol in Russian sturgeon, the motility value obtained after thawing sperm samples was found low (18–25%); in the fertilization study conducted with the same sperm samples, fertilization percentage was obtained as 72.67% [53].

Compared to other species, sturgeon sperm is one of the species with low sperm density due to the mixing of sperm with urine and the originality of the maturation

process of sperm. This low concentration of sperm in sturgeon fish is especially important for the optimum dilution rate in sperm cryopreservation studies where the dilution rate is important. It has been reported that the percentage postthawed sperm motility in Sterlet sturgeon (*A. ruthenus*) depends on the sperm concentration in the samples. While the highest motility after thawing in the study was found in the frozen sperm samples at concentrations of 0.2–1 × 10^9 spz/mL; the sperm concentration of 3 × 10^9 spz/mL, which is higher than the natural sperm concentration in the sterlet, has been reported as suitable for use in cryopreservation procedures as sperm fertilization ability remains at a high level despite a significant decrease in sperm motility percentage. And these findings support the conclusion that high utilization of sperm concentration before freezing may be useful for reducing the volume of sperm retained during freezing and reducing the sample volume required for artificial insemination [54].

In addition to sperm concentration, there are studies investigating the effect of various sperm volumes during freezing. From this point of view, some research was carried out to examine the effect of various volumes (0.5, 0.75, 1.5, and 2 ml) and also the possibility of using the method of vitrification of sperm under deep low-temperature cooling in Russian and Siberian sturgeon. In this study, it was observed that the highest percentage of motility and motility duration was in samples frozen in 0.5 ml Eppendorf tubes. Also, in the study, the following was reported, when cryopreservation of seminal fluid in larger test tubes (0.75, 1.5, and 2 ml), the results were slightly worse [55].

One of the newest issues being investigated in the freezing of sturgeon sperm is the use of ultrasonic waves, which allows the creation of optimum conditions so that the sperm can be preserved at a low temperature. The new methodological approach to low-temperature preservation of fish germ cells using acoustic-mechanical effect offers great opportunities to create new effective deep-freezing methods. The report on the acoustic-mechanical effect on sterlet sperm, it was showed that different parameters of time, frequency, and wavelength can have both positive and negative effects on the reproductive qualities of thawed sperm. It was observed that an increase in the exposure time above 2 min and a frequency up to 5 kHz and a change in the wavelength lead to severe cell damage after defrosting [56].

One of the other current issues studied in sperm cryopreservation in sturgeon is the effect of organotin components (OTs) on fresh and frozen sperm. It has been reported that the accumulation of OTs in the gonad in Russian sturgeon is a stress factor affecting the cells in the cryopreservation process and also this buildup may cause in vitro oxidative stress in sturgeon sperm, reduce gamete quality, and affect fertilization success [57].

2.3 Eel sperm cryopreservation

Eels are species that contain species of economic importance for fisheries and aquaculture, and have reduced natural stocks such as sturgeons. Since the 1980s, natural stocks have been reported to have decreased by 90% for the populations of European eel (*Anguilla anguilla*) and Japanese eel (*A. japonica*) due to climate change, habitat degradation, pollution, parasite infection, and overfishing [58]. All two temperate eel species have been included in the Red List of the International Union for Conservation of Nature (IUCN) as threatened due to population decline, with *A. japonica* categorized as "Endangered" [59] and *A. anguilla* included as "Critically Endangered" [60], which is the highest category before extinction rating. As mentioned earlier, one of the most common methods of protection in endangered species and species where there are difficulties in the reproductive cycle, such as eels,

is cryopreservation. Sperm freezing in eels was first tried in Japanese eel in the 2000s [61]. And after this study, a lot of studies have been done on freezing the sperm of eels, including the sperm of European eels, with the development of protocols being prioritized [62].

The protocol first developed for sperm freezing of eels was the protocol in which DMSO was used as a cryoprotectant in both species. In cryopreservation study using DMSO, post-thawed motility was 45% and above in Japanese eel; in European eels, it has been reported as 35% and above [61]. Although the post-hawed motility value is considered high; because sperm cells frozen using DMSO gives low rates of fertilization rate, and at the same time, with recent studies, DMSO causes epigenetic changes in eel sperm; the use of methanol instead of DMSO as a cryoprotectant substance in the freezing of eel sperm has become widespread. And studies have shown that the use of methanol as a cryoprotectant in the freezing of both Japanese and European eel sperm has led to an increase in motility values after thawed [61, 63]. In another study conducted on European eel; with the use of 5% egg yolk with methanol (10%) as cryoprotectant, it was reported that the motility after thawing was higher than 50%. And this value was found to be significantly higher than the values obtained by using 10% methanol used as a control group in the study [64].

In a study with Japanese eel; artificial seminal plasma (in mM; 149.3 NaCl, 15.2 KCl, 1.3 $CaCl_2$, 1.6 $MgCl_2$, and 20 $NaHCO_3$, buffered with 20 mM TAPS-NaOH at pH 8.1) and methanol (in 1:100 ratio) and 10% methanol in v/v final concentration were tested as extenders and cryoprotectants for sperm freezing. As a result of the fertilization study, although the embryos obtained with cryopreserved sperm had a low survival rate and a high malformation rate; it has been reported that this freezing procedure can be used successfully [65]. In another study conducted on a Japanese eel, three different cryoprotectants were tried to freeze sperm cells stored by creating artificial seminal plasma. At the end of the study, the most successful result was obtained by using 10% and 15% MeOH, in addition to the combination of 5% MeOH and 5% DMA; however, DMSO in artificial seminal plasma has been reported to have no cryopreservation properties and is toxic to sperm [66].

The latest protocols for sperm cryopreservation of European and Japanese eel use methanol as cryoprotectant and they have been adapted to large volumes. In the case of the protocol for Japanese eel sperm, successful fertilization has been achieved and with similar survival rates as with fresh sperm. Moreover, the morphology of the larvae produced with cryopreserved sperm was similar to larvae produced from fresh sperm. In the case of the protocol for European eel sperm, the latest protocol has not been tested for fertilization trials yet, but the motility of frozen–thawed sperm obtained was over 50%, which is the highest ever obtained in this species [67].

2.4 Salmonid sperm cryopreservation

The Salmonidae family consists of important species produced in the world, and total Salmonidae production accounts for <1.8% of the total share of global production [68]. There is an increase in water temperature due to global climate change. Salmonid populations distributed in cold waters are the most studied taxonomic group due to their low tolerance to fluctuations in water temperature. These temperature fluctuations are also thought to affect their reproductive performance [69]. In order to support natural stocks and be used in aquaculture, sperm cryopreservation studies are carried out intensively on Salmonid species. Moreover, the wide distribution of fish species belongs to the Salmonidae family in the world and the fact that

they have been produced for many years has made the species of this family suitable for cryopreservation studies. In the sperm cryopreservation studies conducted to date, post-thawing motility parameters and fertilization rates may vary due to reasons such as differences in freezing procedures, genetic differences in species, and differences in culture conditions. For these reasons, the inability to achieve standardization is one of the most important problems in this field.

The issues summarized below about cryopreservation studies; it will help to understand why standardization on fish-species-specific basis cannot be achieved.

Sperm motility parameters after thawing straws containing cryopreserved sperm; fertilization and hatching rate, straws volume, chemicals, cryoprotectants, spermatozoa density, and reproductive season can be affected by factors. One of the first steps in starting cryopreservation is the choice of materials to be used. In cryopreservation of fish sperm, 0.25 or 0.5 mL straws are usually used. In the selection of the straws to be used; it should be ensured that it is in a volume that will not reduce the motility rate after thawing and will allow the fertilization process to be done easily. When using straws of 0.5 mL; it is preferred because sperm motility parameters after thawing are high and save time during fertilization (*Salmo salar*, [70]; *Salmo trutta* m. *trutta*, *S. salar*, *Salvelinus fontinalis*, S.t. m. *fario*, [31]). In addition to the straws volume change, the glucose rate used in the extender (as it changes the osmotic pressure value) affects sperm motility [31]. In the extender of sperm cryopreservation of salmonid species, sucrose, trehalose, and glucose [71–73] are used, however, mostly glucose is preferred [22, 31, 70, 71, 74]. Sperm concentration in the straws also affects sperm motility parameters. However, this sperm concentration may even differ between species belong to the Salmonidae family. In rainbow trout ($0.5–1.0 \times 10^9$ spz/mL) [75]; sperm concentration in straw, where the survival rate after thawing is the highest, is significantly lower than in other Salmonid species ($2.0, 3.0, 4.0 \times 10^9$ spz/mL; *S. fontinalis,* S. *trutta*, *S. salar*, respectively) [31]. Due to cryopreservation process, cells are subjected to stress due to imbalances in low temperature and osmotic pressure. In order to reduce this stress on the cell and to protect sperm cells from freezing effects, various cryoprotectant (can/cannot penetrate into the cell) agents are used. In 2017, in ref. [76] listed cryoprotectant agents under the headings "Alcohols and Derivatives; Sugars and Sugar alcohols; Polymers, Sulfoxides, and Amides; Amines".

Although the success in fertilization and motility rates with cryopreserved fish sperm to date has been achieved with 10% DMSO [38]; in *S. salar*, the motility and fertilization success rate is higher in 10% methanol than 10% DMSO [77]. Although the egg yolk used in the extender creates difficulty in use due to its viscous structure after thawing, it is a popular cryoprotectant (not penetrate into the cell) used for freezing and storing sperm of various species. The addition of egg yolk and sucrose to the extender together with cryoprotectant agents with penetrating properties into the cell significantly improves sperm quality [78]. Various antioxidants are used in the extender to prevent lipid peroxidation and Reactive Oxygen Species (ROS) activity that may occur during cryopreservation [22, 79, 80]. The addition of α-tocopherol and ascorbic acid to extender lead to a decrease in membrane lipoperoxidation and O2 – i production of *S. salar* spermatozoa, thereby increasing the fertilization capacity [80]. The addition of ascorbic acid to the extender in *S. salar* increases the cell integrity and sperm function of spermatozoa [80]. A decrease in sperm motility after thawing can be associated with membrane permeability and DNA damage. ROS, which affects sperm motility in cryopreservation studies, may also induce lipid peroxidation in the membrane. This can lead to the induction of cell apoptosis [81]. In salmonids, there is a positive correlation between the mitochondrial membrane permeability of

cryopreserved sperm and fertilization [70]. The initiation of sperm motility and the duration of motility depend on the ATP provided by mitochondria in most fish [82]. Therefore, any damage to the mitochondria has a negative effect on motility [70] and can limit the motility and fertilization potential of spermatozoa [83].

Potassium ion has an important place in cryopreservation studies because it has the ability to inhibit sperm motility of Salmonid species based on this inhibitory property, the addition of potassium ions to the diluent has a species-specific effect on Salmonid sperm motility after thawing. Although potassium ion negatively affects the percentage of motility after thawing on the species *O. mykiss*, *S. trutta*, *S. fontinalis*; it showed a positive effect on sperm *Coregonus lavaretus*. This effect of the potassium ion is thought to be due to the osmotic pressure, not the concentration used [84]. This has created a similar situation, such as glucose's ability to change osmotic pressure [31]. In sperm during reproductive season and outside the reproductive season, sperm parameters may differ after thawing in sex-reversed rainbow trout [85], however, it is also stated that sperm collection season does not affect post-thawing motility [71]. These different results in the studies depend on the difference in the extender contents used in cryopreservation, the differences in fish strains [71], and the interaction between the extender and the cryoprotectant substance [86].

One of the goals of cryopreservation applications is that this application is commercialized. For this reason, the creation of a cheap and easily prepared cryomedium will be one of the most important factors in the spread of the application. In addition, starting from the studies aimed at spreading the use of natural products in many areas today, non-chemical methods that have the opportunity to be standardized in sperm cryopreservation can be turned to. This allows the use of minimal chemicals in cryomedium to be emphasized. A prototype of the magnetic field that already exists in nature can be created in the laboratory with the help of a magnet, and the motility parameter values of sperm cells can be increased [87]. It has been suggested that the magnetic field may have an effect on the permeability of the sperm cell [88]. Magnetized sperm or water can be used in cryopreservation trials.

2.5 Catfish sperm cryopreservation

Catfish, which are accepted to have more than 3000 species in the world, show a rapid development among cultured species. However, due to the increase in feed and fuel prices, the production of these fish is also adversely affected. In order to increase the production of these species, hybrid species (with specialty fast growth, disease resistance, and efficient growth rate) are obtained. Since the small number of male fish is asynchronous and the killing of fish is mandatory for sperm collection, sperm cryopreservation provides an important opportunity for these species [89]. Studies have also been carried out to increase sperm motility values and fertilization capacity after cryopreservation in catfish. The rate and temperature of thawing straws after cryopreservation are also parameters that affect sperm motility. In *Ictalurus furcatus*, it is the thawing temperatures that give positive results in motility parameters of 7 and 20 s at 40°C and 40 s at 20°C when using 0.5 mL straws [89, 90]. When straws were thawed for 20 s at 20°C or 40°C, it was observed that the sperm motility results after thawing were similar [89]. In addition to the use of small volumes of straw, catfish sperm cryopreservation was performed in large volumes (1 L) bags using dairy cryopreservation technology, and this method was successfully adapted to catfish [90]. Like studies with salmonid species, egg yolk has been used for sperm cryopreservation in catfish. In *Clarias gariepinus*, 10% egg yolk prevented sperm cell

damage during cryopreservation and thawing processes. It also showed protection against adverse environmental conditions such as temperature, pH and osmotic pressure changes, and against the accumulation of harmful substances caused by the toxicity of cryoprotectant substances. Although fertilization and hatching rates can be achieved in *C. garipionus* without the use of intracellular cryoprotectant agents, this rate is low compared to the use of intracellular cryoprotectant agents such as DMSO [91]. In the extender (365–385 mOsm/kg) in which 10% egg yolk was used with glucose or NaCl in *Silurus triostegus*, cryopreservation was successful in the evaluation of motility parameters after thawing. Necrotic cells were observed in the use of glucose-containing extenders (325 mOsm/kg) with low osmotic pressure [92]. In order to develop standardized protocols for sperm cryopreservation, knowing the sperm concentration is important for the viability rate to be obtained after thawing [89]. This, in turn, can greatly improve the effectiveness of cryopreservation sperm use during the fertilization process. In most of the cryopreservation studies of catfish sperm, motility after dissolution was similar to 1×10^8 spz/mL if 1×10^9 cell/mL was used. It was observed that sperm solutions became viscous at a concentration of 1.7×10^9 spz/mL [89]. Cryopreservation; in the processes of cooling, freezing, and thawing, some biophysical and chemical events occur, such as osmotic changes, dehydration and rehydration, changes in cell volume, formation of ice crystals, and toxicity from cryoprotectant. Sperm cells, which have different characteristics specific to the species, are sensitive to these changes. Therefore, consensus should be achieved between species-specific cryopreservation protocols. An increasing number of studies explaining methods of cryopreservation of sperm in many species are proving this diversity [12]. Cryopreservation has been shown to have detrimental effects on the plasma membrane, mitochondria, chromatin structure, osmotic control, and spermatozoa motility [93, 94]. The cryopreservation process can lead to apoptosis and mitochondrial dysfunction [95], and studies have been carried out at the molecular level in recent years to determine the effect of cryoinjury [96, 97]. After thawing, sperm motility parameters in most species, including *I. furcatus*, show a decrease. This reduction in motility parameters can reduce fertilization potentials and three times higher oxidative stress level has been determined. It indicates that sperm quality may deteriorate after cryopreservation due to a 4-fold increase in the DNA fragmentation level of sperm after thawing [96]. One of the effects of cryopreservation on sperm cells is the increase of apoptotic cells [98, 99]. In order to reduce these effects, "amide" has been used in recent years as a cryoprotector for the protection of sperm [100].

Cryopreservation increases the oxidative stress level and DNA fragmentation of the sperm and thus decreases the sperm kinematic parameter values. Transcriptome analyses are also performed to determine the cryodamage caused by cryopreservation in sperm cells. In these analyses, upregulated genes were identified in sperm samples after thawing and an increase in oxidative phosphorylation activities leading to excessive production of ROS associated with cell death was detected. Despite these negative results, the presence of the potential of sperm to fertilize eggs after thawing is expressed in the fact that compensatory processes occur in the gene expression of sperm cells after thawing to offset these harmful effects (MnSOD, induction to control ROS production; correction of misfolded proteins; apoptosis, functions related to amide biosynthesis) [96]. Sperm cryopreservation can affect several biological processes, including apoptosis, spermatogenesis, mitochondrial activity, ROS production, amide biosynthesis, protein folding, and degradation [96]. Therefore, the effect of cryopreservation is quite complex, with both harmful and compensatory effects on

sperm quality. In addition to the level of gene expression, cryopreservation can also affect DNA methylation, which has been identified as 1266 differentially methylated genes in sperm methyloma [97].

3. Germ cell cryopreservation

Freezing and storage of gametes is used to protect endangered species as well as to ensure sustainability in aquaculture applications. While only sperm cryopreservation is performed in fish, germ cell cryopreservation, which has the feature of differentiation to both gamete types, can be successfully performed. Although cryopreservation and long-term storage of sperm is a technique that has been practiced for a long time, it is impossible to ensure the continuity of generation by using sperm alone. Therefore, germ cell cryopreservation offers an important opportunity in achieving the above-mentioned goals [101].

Germline stem cells isolated from immature gametes can be intraperitoneal transplantation of immunologically immature newly hatched larvae into the body cavity [102]. In addition to the larvae, germline stem cells can be transferred to broodstock fish and embryos [103]. Germline stem cells, transferred to the larvae, migrate to the genital ridge, multiply and initiate spermatogenesis or oogenesis. Cryopreservation of germ cells is performed for transplantation. For this purpose, in cryopreservation germ cells; slow freezing and vitrification methods are used. After transplantation of germ cells, frozen by both methods, to the larvae, there is no significant difference in the rate of migration to the genital ridge and their reproduction compared to the control group [104]. In addition to cryopreservation of all testis tissue, germ cell isolation can be performed from immature gonads after dissection of fish. Freezing of all testicular tissue; while it is made using immature gonads that are frozen in a freezer and stored without the help of exogenous cryoprotectant [105], it can also be done using cryoprotectant [106].

Germ cell isolation in fish can be performed from all cryopreserved testicular tissue as well as from immature gonads. The differentiation of spermatogonia to the ova after the transfer of spermatogonia isolated from cryopreservation testis tissue to the female recipient fish provides a definitive solution to continuity of the species. As a result of isolation from rainbow trout testis tissue in the absence of cryopreservation and the presence of dead fish, transplantation efficiency was found to be 90.61 ± 5.26%, 82.22% ± 11.76%, 73.33% ± 3.33%, and 6.68 ± 6.66%, respectively [107].

In the cryopreservation of oogonia; DMSO, methanol, glycerol, ethylene glycol, and egg yolk are used as cryoprotectants. Between these, the use of DMSO, which is a cryoprotectant that has the ability to penetrate into the cell, gives the best rate on both motility and fertilization rate. Although egg yolk does not have penetrating properties, success has been observed in its use with lactose [108]. DMSO; has been identified as the cryoprotectant substance with the most successful results for cryopreservation of spermatogonial stem cells of *Oncorhynchus mykiss*, type A spermatogonia of *I. furcatus* [109], ovary of *C. carpio* [110], and oogonia [108].

In sperm cryopreservation, evaluation of motility parameters without determining the post-thawing observation and hatching rate will be incomplete in terms of determining the success of the experiment. In addition, the development of germ cell cryopreservation procedures without germ cell transplantation does not yield results. Since spermatogonia/oogonia has sexual plasticity (the ability to produce both eggs and sperm), these mitotic germ cells can be stored by freezing. This application will

be an alternative to sperm cryopreservation as well as freezing and storing fish eggs or embryos [111]. Reducing the complicated steps in germ cell cryopreservation as much as possible (reducing the use of chemicals, applying some of the experimental stages in fish farms, and easy transferability of samples) will accelerate the conservation of species, which is an urgent global problem.

4. Cryopreservation of fish embryo

It is an important issue to use gametes obtained by aquaculture to support natural stocks and, to make cryopreservation techniques that provide long-term protection of these gametes available in all species and reproductive cells of species. Sperm cells, due to its small size and greater durability during freezing, have given more successful results than other reproductive cells, and these features have made sperm the most researched cell in the cryopreservation of fish gamete. Freezing sperm cells is successfully practiced in many fish species, and a protocol has been established for almost cultured fish [38]. However, still, egg freezing has not been successful because of its features such as dehydration problems, large volume, and different membrane permeability. For this reason, many studies have focused on freezing fish oocytes and ovarian follicle. The reasons for the increase in oocytes and ovarian follicle cryopreservation studies are that these cells have a small volume, high membrane permeability, membrane systems are simpler and less sensitive to freezing [112, 113].

The cryopreservation of embryo, which allows the storage of both the female and male genomes, has been a challenging subject of cryopreservation studies for many years. In terms of aquaculture, successful fish embryo cryopreservation will significantly facilitate the establishment and management of genetic selection programs in fish farms [38]. Fish embryos have a low surface-to-volume ratio. It also has a large volume of yolk and a low rate of membrane permeability. In addition to these features of fish embryos, their high sensitivity to low temperatures has made it difficult to use and achieve success in cryopreservation studies [113, 114]. However, despite all these difficulties, there are studies on embryo freezing in fish. Studies have been carried out on chilling and cryopreservation of embryos in 20 different teleost fish [115–117]. The first study on cryopreservation fish embryos (slow-freezing method) was tried and successfully recorded in carp fish embryos in 1989 [118], however, a complete standard has not been established yet.

One of the important issues in the cryopreservation of fish embryos is the toxicity and penetration of the cryoprotectant to be used. In addition to the freezing procedure in the cryopreservation of fish embryos, there are also studies on the selection of cryoprotectants to be used [38]. In an embryo-freezing study with zebrafish, known as model fish, methanol was found to be more effective cryoprotectant compared to DMSO and Ethanediol [119]. In another embryo freezing study using vitrification in zebrafish, it was reported that the embryos survived for 3 h after thawing [120]. In Ref. [121], it was observed that continuity in the development seen only 2.96% seven-band grouper (*Epinephelus septemfasciatus*) embryos, postvitrification. In a recent review study on fish embryo freezing, the issues that need to be developed in relation to the vitrification method that is widely used and tried in embryo freezing are systematically given. In this review, the issues that need to be considered for the development of vitrification protocol are listed as cryoprotectant toxicity, developmental stage of the embryo, and the conditions at the time the embryo to be frozen is treated with cryoprotectant and vitrification [122]. Most of the studies focused

on fish embryo vitrification within the area of toxicity of vitrification solutions. In 2006, it was reported that Japanese flounder (*Paralichthys olivaceus*) embryos were successfully cryopreserved by using vitrification method [123]. Fish embryos show different sensitivity to cryoprotectant permeability at each developmental stage. With this feature that embryos have, some studies have been conducted using various storage methods to determine cryoprotectant flux or concentration at different stages during embryo development [122]. In study conducted on carp, it was reported that the hatching rate was 41% if sucrose was used to protect carp embryos in −4°C. In the same study, it was reported that the use of sucrose and methanol together gave the best results [124]. Recently, in the embryo freezing study, where two different cryoprotectants (DMSO and Methanol) were tested in carp, it was stated that the use of two cryoprotectant substances together had a protective effect by keeping the embryos at −2°C for 1 day and the average larval survival rate was 12.38% [125]. Another study showed that 9.7% of the embryos continued their lives for 2 h by freezing the embryos in liquid nitrogen (−196°C) in *Epinephelus moara* [126]. In a study with carp, using Modified Haga's solution; the toxicity of DMSO and glycerol and its effect on the survival rate of embryos were examined. In the same study where the effect of embryo freezing on the survival rate of the application of different developmental stages was investigated, it was stated that carp embryos were successfully frozen (−196°C) [127].

In cryopreservation of fish embryo studies, the highest success achieved so far has been seen in Persian sturgeon (*A. persicus*) with a hatching rate of 45.45%. In this study, where the vitrification technique was applied, DMSO was selected as a cryoprotectant [128]. Another study whose results were successfully stated was the one which propylene glycol was injected into the zebrafish embryos in freezing and the samples were frozen in liquid nitrogen with a rate of 90,000°C/min. In this study, the survival rate of the thawing embryos was found to be 10% after 24 h post-thawing [129].

Despite all the studies and efforts made on the freezing of fish embryos, unlike fish sperms and germ cells, the work done to prevent crystallization and biological damage during freezing/thawing of fish embryos is still a challenging topic. However, recent studies with cryopreservation primordial germ cells look promising when storing both female and male genomes. Such studies can be used in cryopreservation studies until an undisputed result is obtained in terms of embryo freezing in fish and serve the purpose of cryopreservation.

5. Prevention of disease transmission

5.1 Contamination during the fish gamete cryopreservation and thawing process

The cryopreservation method is one of the assisted reproductive techniques (ART) and has been in use for many years to serve needs such as infertility treatment or genetic improvement and preservation or transportation in living things. Performing this method under aseptic conditions is one of the important factors in the preservation of gametes without microbial contamination and resulting in successful fertilization [130, 131].

The main sources of contamination encountered in cryopreservation studies are (1) Dewar, (2) Cell, (3) Liquid Nitrogen (LN), and (4) Handling (extender, supply, etc.) [131–134]. When the LN container is opened, the upper part begins to cool, and the water in the air turns into ice crystals with a high electrostatic charge, catching the

microorganisms in the air. It is known that these ice crystals fall into the LN container and cause the accumulation of microorganisms in masses at the bottom and can combine with cell residues in the environment. In a study conducted on LN container that was used continuously for 7 and 12 years, it was shown that the contamination intensity did not depend on the year in use, but the microbial diversity was different in each of them [134].

Bacteria are single-celled organisms that have been cryopreserved for extensive research since 1950 [135]. Bacteria are not affected by cold when stored in suitable conditions specific to the species and can reproduce when thawed. For long-term storage at −20°C causes the death of perishable bacteria [136], while bacteria are usually stored in a −80°C freezer with little loss, longer and more appropriate storage is provided with liquid nitrogen and vapor phase (140–196°C) [137]. Cryopreservation is also used for other microorganisms such as viruses and fungi for research purposes [138]. In gamete cryopreservation studies, cryoprotectant agents are added to the extender to protect the cell from freezing. The cryoprotectants and nutritional elements used to store microorganisms at −196°C are very similar to the solutions used to store sperm cells. Therefore, while the gamete cells are frozen, microorganisms can be frozen together with the cells unintentionally [139–141].

Also, infected gamete samples can contaminate other pathogen-free samples when stored in the same LN container. Especially in human sperm storage conditions, cross-contamination poses a major problem. Storing the sperm of an individual carrying the disease and the sperm samples of an individual who is not diseased in the same LN container causes a healthy parent to have a diseased child. It has been shown in studies that liquid nitrogen can contaminate the samples to be placed in contaminated with bacteria and viruses [142].

As we mentioned above, during the transfer of LN used in cryopreservation, microorganisms present in atmospheric air can contaminate liquid nitrogen [134, 143]. Although the fact that many microorganisms in the air are not fish pathogens does not seem to be an important problem in terms of disease transmission, the presence of bacteria in the environment where sperm is present is an important factor that impairs its quality [144]. In other words, microorganisms use the nutrients and oxygen in the environment, which are necessary for the survival of the sperm, as well as changing the pH of the environment [8], and this affects the quality of the sperm and changes its fertilization capacity [145]. In addition, the most important problem will be the use of gamete cells contaminated with the microorganism in fertilization, the transmission of pathogens to the embryo, and the emergence of diseased offspring [146].

5.2 Disease transmission via gametes to embryo

Research that has been conducted on sperm and embryo has found a relationship with pathogens such as viral, bacterial, fungal, and parasitic organisms [147]. It has been reported that some of them are in the seminal plasma, attach to the sperm cells, but do not enter the cell, and attach to the egg from the zona pellucida [142, 148].

To summarize briefly, the literature on vertical transmission of fish pathogens; It has been proved that the infected pancreatic necrosis (IPN) virus is present in the ovarian fluid and can be attached to the salmonid sperm and transmitted vertically to the embryo [149–152]. Viral hemorrhagic septicemia virus (VHSV) and Infectious hematopoietic necrosis virus (IHNV) have been isolated from the seminal and ovarian fluid of salmonid species [153, 154]. In addition, some bacteria have been shown by staining sperm samples, and *Aeromonas* sp., *Pseudomonas*

sp., and *Flavobacterium* spp. have been detected in the seminal plasma of trout using culture methods [144, 155, 156]. *Renibacterium salmoninarum* is bind to tail of sperm and has been found intra-ovum of rainbow trout [146]. In a study showing that *Piscirickettsia salmonis* can be found in the seminal fluid of trout, it was shown that vertical transmission is also possible [157].

5.3 Eliminate non-cellular disease agents from gametes

It has long been known that semen can contain a variety of viruses, and good safety protection systems and methods have been established in many laboratories to reduce virus particles from semen obtained from humans with viral diseases [158]. Viral Infection is recognized as a possible cause of male infertility in humans [159]. Among HIV-infected men, vertical transmission to infants has been reported when in vitro fertilization is performed after sperm washing [160].

However, as far as we know, there is no study on the inhibition of the transmission of viruses from sperm to embryo in fish. It is known as general information that the viral titer decreases after the freezing–thawing process [146], so the viral load in the sperm can be lowered by cryopreservation.

5.4 Antibiotics

Although antibiotics are used to eliminate bacterial contamination in sperm freezing, it should also be considered that they are harmful to sperm [8, 161]. Although there are studies in which antimicrobial biomolecules (resveratrol, curcumin, etc.) are used instead of antibiotics, these have not yet taken the place of antibiotics [162].

5.5 Sperm washing and cell separating methods

Density gradient centrifuge method is a method that allows cells to be separated according to their size. Although this method is frequently used for sperm washing in mammals, that is, for removing microorganisms, its applications in fish are generally based on separating quality sperm cells from all other foreign substances [163]. Although the "swim-up" method, which is one of the sperm washing methods, is also used in mammalian sperm cells, this method does not work in fish since motile sperm must be used in this method [155].

During the process of sperm washing, freezing, and thawing, in order to obtain a high concentration of quality spermatozoa, it is critical that we reduce and remove any risk or chance of pathogen contamination during the preservation of frozen sperm in tanks. While sperm washing is usually performed before cryopreservation of mammalian sperm [164], in sperm washing studies applied in fish, since 2010, the washing process is performed after thawing [165–167]. In some species, it is used to separate seminal plasma from cells by centrifugation before freezing [168]. In the study in Ref. [168], firstly, seminal plasma was separated by centrifugation, and then live sperm cells were separated from the dead by magnetic selective. As a result of this study, it was possible to obtain better quality and functional sperm.

Studies using sperm washing method in fish have increased, especially in recent years. In the first study on this subject, in which frozen sperm from carp were used, it was determined that the sperm washed using the percoll gradient method had high motility, and the spermatozoa, which were immobile and whose membrane was damaged during the freezing–thawing process, were also removed. As a result, it is stated

that the use of this technique will allow easier and higher quality spermatozoa to be obtained compared to other biotechnological cell separation methods [169]. Another study conducted in sturgeon includes the evaluation of sperm motility parameters and sperm characteristics after gradient centrifugation method of control and frozen sperm samples [165]. In a freeze–thaw washing study performed with testicular sperm, the fertilization rate was found to be higher than the control [170]. In the only study in which sperm washing was used to remove bacterial load, it was observed that gradient centrifugation method reduced bacterial load, although it caused a decrease in motility rates in rainbow trout sperm [155]. Again, according to the results of the study by the same authors on the relationship between newly completed sperm washing and fertilization success, low motility after washing did not significantly change the fertilization success and survival rate (unpublished data).

In studies using the density gradient centrifugation method, percoll was the most widely used gradient-forming chemical [169, 170]. Sil-Select Plus TM [155] and AllGrad® 90% [167], which are commercial solutions produced in humans, have been tried in fish and successful results have been obtained.

5.6 Disinfection of gametes and embryo

When the sperm are treated with a disinfectant, the sperm cell loses its vitality. However, iodine and anti-fungal treatment of eggs with formaldehyde is routinely and repeatedly used during the eyed stage of salmonids [146, 171]. Disinfection of eggs with iodophor prevents some viruses (VHSV, etc.) and bacteria (*Flavobacterium psychrophilum*, *Yersinia ruckeri*, etc.) from passing into the embryo, while it cannot provide protection for others (IPNV, *R. salmoninarum*, etc.) [146]. According to the literature, no studies were found regarding the presence or solution of contamination in frozen embryos or eggs (perhaps because embryo and egg cryopreservation is a new subject).

5.7 Suggestions

In cryopreservation facilities, it has been reported that the microbial load should be controlled before freezing sperm in order to prevent cross-contamination in liquid nitrogen [172]. Freezing sperm from brood stocks that are not infected or have the least pathogen in their sperm will be the best solution. This can be possible with continuous health checks and monitoring of fish. The use of vaccinated brood stocks to prevent the spread of the disease with high mortality would be excellent for sustainability. Sperm cells of brood stocks infected with a possible pathogen can be separated from the pathogen with the sperm washing method and then frozen. This is also promising technique in obtaining quality sperm. In addition, continuous disinfection and use of tanks, UV sterilization of liquid nitrogen used in small volumes, sterilization of all materials used, and sealing the ends of the straws by burning will prevent possible contamination [133].

Establishing special sperm collection laboratories for cryopreservation would also be a good solution to prevent contamination. The use of a common laboratory with professional staff, where special Biosafety measures are taken and hygiene rules are followed, will be beneficial for every fish farm [131]. There are cryobanks where gametes are stored in different countries, but since the number is not sufficient, it would be beneficial to create an easily accessible cryobank for each country [38].

Cryostorage facilities are expensive and may not be affordable or readily available due to their constant need for electrical power and liquid nitrogen production [173]. Additionally, monitoring liquid nitrogen levels is a time-consuming and expensive process that requires constant visual inspection. Therefore, cryostorage systems have recently been said to be prone to failures leading to undesired sample losses [174]. Liquid nitrogen is seen as a pathogenic transmissible agent, and its production is also a high carbon footprint procedure [173]. The most convenient way to prevent LN contamination is to use filtered (sterile) LN. However, this application is not suitable for use in practice due to its high cost. It is recommended to find a cheaper, simpler, greener alternative [173]. Another alternative is to use ultra-low freezers at −150°C, which has increased production today, instead of liquid nitrogen tanks, and it is thought that the risk of nitrogen vapor contamination can be reduced [175]. And even in a recent study in zebra fish, an ultrafreezer of −150°C was used for sperm cryopreservation and successful result was achieved [176].

It has also been reported that freeze-dry technology can eliminate the difficulties and contamination risk of cryo-storage using liquid nitrogen in today's technology [173, 174]. In fact, some mammalian spermatozoa have been studied with this method [173, 174, 177]. In these studies, sperm motility is not important and efforts are made to prevent DNA damage of sperm. Therefore, this method can be used for large mammals in the intracytoplasmic sperm injection (ICSI) method, but it does not seem possible to apply it to animals with multiple eggs such as fish [178]. In the literature, there has not yet been a study in which this technology is used in fish species.

Although the OIE has a "Health code" for terrestrial animals on the prevention of the risk of transmission of cryopreserved sperm-borne disease, there is no such information in the "Aquatic Animal Health Code" [171]. In the future, it will be useful to come up with standard methods for aquatic organisms.

6. Conclusions

Cryopreservation applications are one of the important assisted reproductive techniques that can be used to maintain the vitality of an organism. When the subject is to ensure the sustainability of a living thing, external factors such as its own biological, physiological, genetic, and epigenetic characteristics as well as its feeding regime, living environment, and presence of pollutants are also effective. For this reason, standardization of all these differences while conducting research is very difficult (perhaps impossible with current practices) and requires long time-consuming studies. However, in order to eliminate the negative effects of cryopreservation on the cell, the standardization of the factors mentioned above, as well as the laboratory environment, ensures the quality of the cells to be obtained. In reproductive biotechnology studies, laboratory studies should be carried out under sterile conditions after obtaining eggs and sperm from the fish broodstock.

Ensuring the importance shown in aseptic laboratory environment also allows the cells obtained to increase the viability rate of the offspring, as well as to be healthy. Fish farms carrying out these practices should be certified and sample entry from non-certified farms should not be allowed. A living organism is in constant contact with its environment and is constantly under this influence. Breeding and fish health practices are subjects that cannot be considered independently of each other. In this context, if it is desired to ensure the continuity of high quality, fast growing, and healthy generations, it is not possible for these two fields to be independent from each other.

Although the concern and necessity of this issue have been stated in the studies, the use of sterile environments has not been actively provided yet. Informing the people working in fish production farms on this subject and organizing meetings/seminars that emphasize the importance of the subject and organizing trainings/courses would be beneficial. In addition, these practices may be made compulsory by various regulations.

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Chapter 2

The Current Status of Semen and Oocytes Cryopreservation

Masindi Mphaphathi, Mahlatsana Ledwaba and Mamonene Thema

Abstract

Assisted reproductive technologies are critical in the preservation of gametes from endangered species. As a result, cryobanking is critical in reproduction facilities for the gametes conservation of endangered species for future use. Furthermore, cryobanking allows for the preservation of genetic variability through biotechnological reproduction programs. If oocyte cryopreservation is successful, the timing of *in vitro* maturation and subsequent to *in vitro* fertilization (IVF) will be possible. Cattle oocytes are very sensitive to cryopreservation due to their complex structure, and they are also very sensitive to chilling, which can harm their viability. During the cryopreservation process, sperm membrane proteins and carbohydrate composition change, sperm membrane structure is disrupted, and sperm viability is reduced. Extenders are frequently required during cryopreservation, for improving sperm cryopreservation technologies and is therefore necessary to have a thorough understanding of the properties of the extenders. Extenders have been enriched with antioxidants such as Glutathione to protect sperm motility and integrity from oxidative damage and the reactive oxygen species produced during cryopreservation can be neutralized using antioxidants.

Keywords: semen, oocytes, extender, cryopreservation, slow freezing, vitrification

1. Introduction

Cryopreservation is the expertise of freezing and cryogenic storage of biological materials at extremely low temperatures, occasionally utilizing solid carbon dioxide at −80°C or more often liquid nitrogen at −196°C [1]. This procedure is essential for preserving gametes and genetic diversity in both known and endangered species. Cryopreservation of gametes and genetic diversity provides numerous advantages as it paves the way for the successful application of current biotechnologies like cloning, transgenesis, and long-term storage/conservation of animal genetic resources [2]. Oocyte cryopreservation is beneficial for the treatment of infertility and has broader clinical implications than embryo cryopreservation [3]. However, for the quality of the gametes or tissue not to deteriorate during long-term storage, a good and reliable methodology is required. Many reproductive centers have established

cryopreservation methodologies for many species. However, the survival rate of the gametes or tissues has declined over time due to the lower temperature and metabolic reactions of the gametes which are impaired during cryopreservation. Cryopreservation causes the formation of intracellular ice crystals and osmotic stress, which causes cell damage, oocyte quality degradation due to their susceptibility to chilling, and a reduction in sperm survival rate [4]. Cryopreservation is one method of preserving sperm and oocytes *ex-situ*. Furthermore, the cryopreservation process has proven to can reduce the number of cases of extinction. Moreover, the use of semen extenders, medium, antioxidants and cryoprotectants (CPAs) proved beneficial in preserving the gametes. The semen extenders, mediums, antioxidants, and CPAs are nutrients and antibiotics that increase the quality and survival rate of the gametes. It was discovered that successful gametes cryopreservation, requires the use of semen extenders, mediums, antioxidants, and CPAs. Typically, the pace and susceptibility of sperm to subzero temperatures relate to the content of cryoprotective chemicals and membrane-stabilizing additives. Therefore, cryopreservation may be an effective method of preserving fertility as the frozen–thawed sperm may be utilized for intrauterine insemination, IVF, or intracytoplasmic sperm injection [5].

2. History of cryopreservation

This chapter will discuss the current state of oocytes and sperm cryopreservation, as well as their prospects. Mammalian sperm cryopreservation has previously proven to be difficult due to the lack of methodologies that can help sperm and oocytes withstand extremely low temperatures. Whereas cryopreservation has been determined to be an ineffective reproductive medicine preservation treatment since 1970 [6]. To date, the sperm cryopreservation in ovine and bovine species has improved over time. Whereas sperm cryopreservation remains a challenge in mammalian species such as porcine and humans. Furthermore, cryopreservation of oocytes has proven to be difficult in all mammalian species. Several methodologies have been tested on mammalian oocytes, in the past, beginning with the first cryopreservation in the year and continuing to the present [7]. Cryopreservation of oocytes in many mammalian species, such as porcine and horses, remains a major challenge.

To achieve the best results during cryopreservation, a thorough grasp of sperm physiology is essential [8]. The fact that sperm are tiny cells with a vast surface area is a crucial aspect of sperm cryobiology [9]. Sperm are less vulnerable to potential harm because of these traits, which impact the intracellular cytosol's viscosity and glass transition temperature [10]. Organelles in the sperm may be destroyed in the absence of cryoprotective substances due to cold shock and the stimulation of ice crystal formation [11]. More research is needed to determine the methodologies that can successfully improve the quality of oocytes after cryopreservation. Cryopreservation of semen and oocytes will aid in the preservation of domestic and wild species' genetic diversity, as well as the dissemination of superior genetics, gene banking, and the extinction of superior and endangered species. Semen cryopreservation is also used in artificial insemination (AI) and IVF procedures. However, previous research has shown that using post-thawed semen for AI results in a lower pregnancy rate. Offspring have also been reported to be born from frozen–thawed oocytes in bovine, ovine, and horse.

3. Fundamentals of the cryopreservation

3.1 Extenders

Semen extenders are liquid diluents added to sperm to preserve its ability to fertilize, and they contain protective ingredients that allow sperm to survive outside the reproductive tract of the male animals [12, 13]. Furthermore, semen extenders protect sperm by stabilizing the plasma lemma, providing energy substrates, and preventing the harmful effects of pH and osmolarity changes over time during *in vitro* storage [14]. When a proper semen extender is added to the sample before evaluation, the accuracy of sperm motility determination may improve [15].

During the chilling and shipping processes, semen extenders act as a buffer to protect sperm cells from their own harmful byproducts as well as from cold shock and osmotic shock [16]. Semen extenders has the ability to prolong sperm storage and transportation, allowing it to be used during AI, IVF and other research studies. Currently, the semen extenders are categorized either as short (approximately 3 days; *in vitro* liquid storage) or long term (approximately 5 days; *in vitro* liquid storage or cryopreservation for years) [17]. For the past decades, some commercial vendors have made around 80% of the semen extenders for porcine sperm readily available [18].

3.2 Cryoprotectants

Cryopreservation methods aim to preserve the viability of tissues and cells by focusing on the mechanisms of harm and protection in living cells and tissues at low temperatures [19]. The impact of subzero temperatures on normally healthy tissue should be recognized to properly comprehend the role of cryoprotective agents. Since water makes up around 80% of tissue mass, both intracellularly and extracellularly, has the greatest impact on the detrimental biochemical and structural changes that are hypothesized to cause cryopreservation injury [20]. Due to the presence of salts and organic molecules in the cells, the freezing point of cell water is substantially lower (even −68°C) than the freezing point of pure water (about 0°C).

Cryoprotectants are divided into two groups: permeable and non-permeable CPAs. Non-permeable and permeable CPAs improve cell survival while decreasing cellular water content to help prevent intracellular ice crystal formation. Permeable CPAs are macromolecules that pass through the sperm plasma membrane. A cryopreservation diluent's functions include providing the sperm with energy sources, shielding them from temperature related harm, and maintaining an environment that allows the sperm to survive for a while. Glycerol, Propylene glycol, and Ethylene glycol (EG) are three examples of permeable CPAs that are commonly used (**Figure 1**). To enhance post-thawed sperm viability and fertility, each of the several media components was studied alone and in combination [21]. The gametes exposed to those penetrating

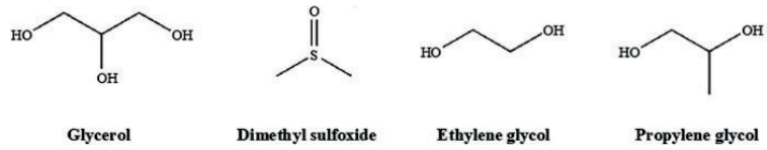

Figure 1.
The penetration CPAs that are widely used: Glycerol (GLY), dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PG). Adapted from Whaley et al. [20].

solutes undergo intense initial dehydration, then rehydration, resulting in a chance of gross cellular swelling [22]. Glycerol at 3% has shown to maintain the cryo survival rate of sperm from different species; thus, larger amounts of permeable CPAs concentrations have shown to lead to more cellular damage [23], while the higher concentrated CPAs are more toxic to oocytes. Glycerol reduces intracellular water freezing while adjusting sperm osmolality via invasive thermal protection [24]. The discovery of Glycerol's effectiveness in preventing various phase transitions while freezing via increased water permeability and fluidity of the sperm membranes resulted from research to understand the mechanisms of CPAs [25]. Using minimum volume methods, a higher cooling rate can facilitate vitrification with less concentrated CPAs, and a higher warming rate will prevent it [26]. Non-permeable CPAs such as sucrose can facilitate dehydration and vitrification, which reduce the required concentration of permeable CPAs [27].

3.3 Antioxidant

During semen cryopreservation and thawing, increased reactive oxygen species (ROS) generation and decreased antioxidant levels were observed. As a result, oxidative stress may have a role in sperm injury during cryopreservation. Oxidative stress is the imbalance between the formation of ROS and antioxidant defenses, resulting in considerable loss of sperm function. Therefore, the ROS generated during oxidative stress can be neutralized by the use of antioxidants. Antioxidants are chemicals and reactions that dispose of scavenging, suppressing, or resisting the creation of ROS. Antioxidants have been shown to inhibit or reduce the lipid peroxidation reaction, resulting in less oxidative stress and damage [28]. Antioxidants and antioxidant enzymes that are found in the seminal plasma of the semen protect sperm from oxidative damage. These antioxidant mechanisms protect the sperm, including enzymatic antioxidants [Catalase, Superoxide Dismutase (SOD), reduced Glutathione & Glutathione Peroxidase (GPx)], [29, 30] and non-enzymatic antioxidant systems [GPx, Vitamin C, E, Cysteine & Glutathione (GSH)]. These antioxidants protect sperm from cryo injuries caused by reactive oxygen species [31, 32]. However, due to the addition of extenders to the seminal plasma during cryopreservation, the quantities of these antioxidants decrease [33]. Therefore, finding low cytotoxic antioxidants at a suitable concentration is very important in improving the frozen–thawed sperm quality [34].

Recent studies suggest that supplementing cryopreservation extenders with antioxidants improves sperm quality in bovine, ovine, porcine, canine, and human, enhancing sperm motility and membrane integrity following thawing [35]. A range of antioxidants is active in the body including enzymatic (endogenous) and non-enzymatic (mainly brought by food) antioxidants. All of them can be intracellular or extracellular antioxidants and can be used during the cryopreservation of semen. To reduce cryodamage, numerous exogenous, non-enzymatic antioxidants were introduced to maturation, vitrification media, and extenders for mammalian sperm, oocytes, and embryos.

4. Methods of cryopreservation

There are numerous cryopreservation methods for cryopreserving semen and oocytes of different species; methods include slow freezing (programmable freezer),

rapid freezing, and ultra-rapid freezing (also known as kinetic vitrification). These methods represent a particular drawback in determining the most appropriate method for cryopreservation [36]. Various processes have been developed for semen and oocytes cryopreservation technology in recent years [37].

4.1 Conventional slow freezing

Slow cryopreservation of semen has been a useful method and is still used during cryopreserving semen and oocytes [38, 39]. A programmable freezer with significant control over the ideal freezing rate is necessary for conventional slow freezing. The temperature progressively drops below the freezing point during cooling, whereas both extracellular and intracellular spaces can generate ice [40]. In order to ensure fine control over numerous elements (such as thermal shock) that lead to cell damage, slow freezing primarily calls for a relatively low concentration of CPA agent, combined with sufficiently slow cooling/freezing rates [41]. In a summary, slow cooling is mixing low concentrations of a penetrating agent like DMSO (usually ≤1.5 M) as well as a non-permeating agent (often sucrose or trehalose, ≤ 0.3 M) with controlled slow chilling rates to gradually dehydrate sperm and oocytes. The sperm and oocytes are kept in liquid nitrogen at −196°C till they are required for usage after cooling to about ≤150°C [20]. One disadvantage of this procedure is the inability to freeze extremely small amounts of semen, as in the case of surgical testicular sperm retrieval [37]. Slow freezing methods using a programmed freezer is traditionally used for oocyte cryopreservation, and these procedures typically take several hours [3], oocytes are progressively chilled over 2 to 3 hours in two or more steps, either manually or automatically with the aid of a programmable freezer [42]. Semen straws can be frozen in huge quantities with the use of programmable freezers, which also allow for regulating the freezing pace. By cryopreserving the semen straws at −80°C for 7 to 15 minutes and then submerging them in liquid nitrogen, these freezers can be used to stimulate pellet freezing [43]. The advantage of several programmable freezers is the ability

Characteristics	Vitrification	Slow freezing
Direct contact with liquid nitrogen	Yes	No
Ice formation	No	Yes
Time	Fast (minutes)	Slow (hours)
CPA equilibration	Yes	Yes
CPA concentration	High (over 40%)	Low (10–15%)
Cooling rates (°C/min)	15,000-30,000	0.15–0.30
Cost	Protocol-dependent (usually inexpensive)	Equipment-dependent (usually expensive)
Special equipment	No	Yes
Technical expertise	Risky	Simple
Routinely applied for cryopreservation of human ovarian tissue	No	Yes

Adapted from: Amorim et al. [7].

Table 1.
Comparison of the characteristics between vitrification and slow freezing methods.

to personalize the freezing curve, for example, 4 to −5°C at 4°C/min, −5 to 110°C at 25°C/min, and − 110 to 140°C at 35°C/min, before submerging the semen straws in liquid nitrogen [44]. According to some theories, the formation of ice crystals during slow freezing raises the electrolyte concentrations inside cells and could harm the sperm and oocytes chemically and physically. Therefore, slow freezing appears to have a lower survival rate than vitrification [3].

4.2 Conventional straw vitrification

Vitrification is another important method for improving the survival rate of cryopreserved oocytes that is both time saving and does not require any special equipment [45]. The most typical process for vitrification includes adding CPAs step by step in cryomedia [20]. Although vitrification's rapid freezing defends cells from the majority of chilling-related harm, including membrane damage, it necessitates the use of hazardous CPAs solutions in higher concentrations [46]. Oocytes are introduced to a solution that contains 7.5% v/v EG and 7.5% v/v DMSO for 5 to 15 minutes during the initial equilibrium phase. The oocytes are subsequently subjected to a vitrification solution containing 0.5 M sucrose, 15% v/v DMSO, and 15% v/v EG. The oocytes are then kept in liquid nitrogen at −196°C after a brief incubation (≤ 1 minute). After gradually removing the CPA, the oocytes are promptly warmed to prevent the development of ice crystals, and then cultured in a culture medium until use [4, 47, 48]. Vitrification solution for embryos must be treated at a low temperature of 4°C [47]. One method for increasing the cooling rate and vitrification is to use liquid nitrogen vapor instead of liquid nitrogen only [49]. The study discovered that the vitrification method involving the use of only non-permeable CPAs for cryopreservation of abnormal sperm samples was an effective alternative to the vitrification method [44]. The differences between the vitrification and slow freezing method are shown in **Table 1**.

4.3 Liquid nitrogen vapor

Semen is poured into 0.25 or 0.5 ml straws, placed on a rack, and frozen in liquid nitrogen vapor. The temperature of which should be determined by the height above the liquid nitrogen after dilution and cooling of the semen samples [21]. When utilizing a styrofoam box, the samples are placed on a rack that is suspended 3 to 4 cm above the liquid for 7 to 8 minutes before the straws are submerged into liquid nitrogen for storage [43]. Alternatively, the freezing height above the liquid nitrogen should be determined by the reported straw size [50]. For storage, it was recommended that 0.5 ml straws be frozen 4 cm over liquid nitrogen for 5 minutes, whereas 0.25 ml straws must be placed 16 cm over liquid nitrogen for 2 minutes, lowered to 4 cm for 3 minutes, and then submerged in liquid nitrogen [21].

4.4 Solid surface vitrification

Solid surface vitrification (SSV) is a cryopreservation technique applied to the preservation of embryos and oocytes. In this technique, which combines many others, oocytes are combined with CPAs and partially submerging a metal surface in liquid nitrogen to pre-cool it to −180°C, SSV employs the metal surface as a cooling template for microdrops of vitrification solution containing oocytes or embryos [51]. The SSV maximizes cooling rates, leaves ample room for tissue, and prevents the formation of gas phase liquid nitrogen bubbles [52]. This technique was initially created for use

with mammalian oocytes [51]. To store the vitrified droplets in liquid nitrogen, they are put into a cryovial. Sperm can be stuffed into tiny capillaries in this experiment. The capillaries can be permitted to be exposed to the cryo-chilly chamber's (−180°C) surface. The benefits of this approach include lessened DNA damage and lessened sperm tail damage [53].

5. Cryo survival rate

The sperm quality after thawing is believed to be influenced by its pre-freezing qualities. The cryo survival rate of post-thawed sperm can also be impacted by pre-freezing semen quality factors, such as sperm motility and the abstinence time of sperm donors [54]. Moreover, sperm with aberrant motility characteristics (asthenozoospermia and oligoasthenozoospermia) are especially vulnerable to cryo-damage, which could lower their fertility [55]. The sperm plasma membrane's lipid content has a significant impact on the sperm's cryotolerance and cold sensitivity. The size, shape, and lipid composition of sperm from various species may vary, which could have an impact on how resistant they are to cryo-injuries [56–58].

Two competing theories attempt to explain why cryopreserving the gametes is harmful. (i) The creation of intracellular ice crystals during cryopreservation causes severe osmotic and physical damage to the sperm, which later reduced sperm functioning [34], and (ii) during chilling, ice crystals form inside the cells, and fatal increases in solute concentration occur in the remaining liquid phase. The cryopreservation and thawing procedure during cryopreservation creates oxidative, osmotic, and thermal pressures, which might compromise sperm quality and lead to a low fertility rate [33]. Despite advancements in cryopreservation technology, the rate of functional post-thawed sperm recovery remains low [59].

The cryo survival rate of oocytes cryopreserve with the use of the slow freezing method ranged from 74–90% [60]. Oocytes are prone to ice recrystallization episodes during storage and thawing [54, 61, 62]. During the thawing process, sperm and oocytes are vulnerable to metabolic damage caused by oxidative stress [63]. The cytoskeleton, lipid droplets, membrane system, and microtubules are the parts of the cell that are most impacted [49]. Oocyte cell membranes resemble female mammalian gametes visually and are much more vulnerable to the effects of cooling than embryonic and even zygotic membranes [63]. Oocyte membranes have a high melting point, making it easier for the lipids to be damaged by a drop in temperature and lose their ability to function as a membrane.

Transzonal processes, tiny microfilaments that keep the meiotic spindle in the proper position during maturation, are damaged by cryopreservation, which has an impact on communication between oocytes and the cumulus cells around them [64]. It was recently discovered that the gamete's lipid and adenosine triphosphate content is influenced by the number of cumulus cells adhering to the oocyte [65]. The decreased survival and development rates of cryopreserved immature and *in vitro* matured oocytes are believed to be mostly caused by this [64].

6. Conclusion

The maintenance of fertility through semen/sperm and oocytes cryopreservation is a crucial component of assisted reproductive techniques, although sperm and

oocytes functions may be negatively impacted by cryodamage to cellular constituents. However, the effectiveness of freezing sperm and oocytes can be improved by comprehending the cellular and molecular alterations. To optimize cryopreservation and thawing methodologies, increasing pregnancy rates in IVF cycles, better understand the role of oxidative stress in the lower developmental competency of cryopreserved gametes, and additional studies are required.

The extenders, mediums, kind of molecules, and concentration utilized for each species samples are all related to the varied effects of each antioxidant supplementation, which improves distinct measures of sperm quality. Antioxidants are increasingly common when semen is being cryopreserved because they may lessen oxidation. Certain antioxidants have been shown to have superior efficacy, while others have less encouraging outcomes. Antioxidant combination, extender concentration, and quality are just a few of the variables that might make adding antioxidants to semen extender during cryopreservation successful or unsuccessful. The development of more effective methods for cryopreservation of cells and the expansion of their clinical applications may be made possible by understanding the underlying chemistry and biology of freezing and thawing processes.

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Conflict of interest

There are no conflicts of interest.

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Chapter 3

Female Fertility Preservation: Different Interventions and Procedures

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Abstract

A human being is made up of two living cells: the egg and the sperm, which pass the torch of life to the next generation. After zygote, the fertilized egg undergoes a series of mitotic divisions. First division into two cells is called blastomeres, and then four cells to 64 cells are called the morula stage. Five days after fertilization, the embryo reaches the blastocyst stage. This blastocyst is attaching itself to the uterine wall for implantation. Implantation is complete when the blastocyst is fully embedded in the endometrium a few days later. Cryopreservation of ovarian tissue, oocytes, embryos, and blastocysts has become an integral part of improving the success of infertility treatment and fertility preservation. Various cryopreservation strategies have been proposed to enhance cell survival and preserve cellular function. It also increases the efficiency of assisted reproductive technology (ART) procedures, enables biodiversity conservation, and provides protection to a valuable biological material. However, successful cryopreservation requires the use of cryoprotectants. The chemical and physical effects of these reagents/processes cause extensive cryogenic damage to the plasma membrane, leading to changes in its normal function. In this chapter, we will discuss different interventions to preserve fertility, including cryopreservation methods and cryoprotectants used.

Keywords: zygotes, embryos, freezing, slow freezing, vitrification

1. Introduction

The unexpected discovery of the cryoprotective properties of glycerol revolutionized the field of cryopreservation and sparked a great deal of further research [1]. However, cryoprotectant toxicity, a fundamental barrier to realizing the full potential of artificial cryoprotection, generally remains a little-known phenomenon.

Successful cryopreservation of sensitive cells, tissues, and organs requires the use of cryoprotectants [2]. Cryoprotectants work by increasing the concentration of

solutes in cells. However, in order to be biologically viable, they must be easily permeable and non-toxic to cells. The toxicity of CPA depends on the inherent properties of the chemical itself. Therefore, the toxicity of cryoprotectants limits the concentration available, thereby limiting the cryoprotective efficiency of these agents [2–5].

Of course, longer CPA exposure time increases toxicity, which may be non-specific toxicity due to the interference of water molecules with cell membranes or specific toxicity due to CPA type and concentration [6, 7].

Gook identified the delicate balance between protection and toxicity associated with the use of glycerol and other cryoprotectants such as propylene glycol and ethylene glycol [8]. However, some cryo-damage is inevitable regardless of the use of cryoprotectants, as each cell type responds differently to cryoprotectants and cryopreservation.

There are two main categories of cryoprotectants: osmotic cryoprotectants and non-permeable cryoprotectants [9, 10].

1.1 Permeable cryoprotective agents (CPAs)

Permeable cryoprotectants include glycerol, methanol, dimethyl sulfoxide (DMSO), dimethylacetaldehyde, ethylene glycol, and propylene glycol. They are low molecular weight chemicals that penetrate the plasma membrane and displace water in cells [11]. However, high concentrations of osmotic cryoprotectants can prevent ice formation during cryopreservation of cells, tissues, and organs at low temperatures. However, CPA becomes more and more toxic as the concentration increases [6].

Cryoprotectants work by increasing the concentration of solutes in cells. However, in order to be biologically viable, they must be easily permeable and non-toxic to cells. The toxicity of CPA depends on the inherent properties of the chemical itself. Exposure time and temperature. Of course, longer CPA exposure time increases toxicity [6].

1.1.1 Glycerol

Glycerol is a non-electrolyte compound, so it can reduce the electrolyte concentration in the remaining unfrozen solution in and around the battery at any given temperature. It has been used for many years in cryobiology as a cryoprotectant for blood cells, animal sperm, and bacteria, which can be stored in liquid nitrogen at low temperatures [12].

In 1937, glycerol was used as a freezing medium for semen from bulls, rams, stallions, boars, and rabbits during the freezing stage (−21°C). About 10 years later, Polge et al. demonstrated the positive effect of glycerol on frozen poultry sperm [1]. However, glycerol is toxic to spermatozoa through protein denaturation, modification of actin interactions, and induction of plasma membrane fragility during cryopreservation [13, 14]. Good cryoprotective effects were obtained when the glycerol concentration was in the range of 0.5–2 M [15].

Therefore, mixtures of cryoprotectants have been shown to be less toxic and more effective than using a single CPA [6].

1.1.2 Dimethyl sulfoxide (DMSO)

DMSO and glycerol are probably the two most widely studied CPAs, although the relative success rates of each generally vary by species [16]. Since the early history of

cryopreservation, DMSO has been the cryoprotectant of choice for most animal cell systems.

Lovelock and Bishop were the first to document the ability of DMSO to attenuate freezing-induced cellular damage during slow cooling of bull semen [12]. DMSO also protects against certain aspects of biological damage and radiation damage suffered during cryopreservation. It is estimated that the hydrogen bond between DMSO and water is about 30% stronger than that between two water molecules [17].

DMSO crosses biofilms with great ease, with minimal evidence of damage, and has extensive solvation properties [18]. In 1988, Friedler et al. showed that DMSO was more effective than glycerol [19].

Nonetheless, its impact on cell biology and apparent toxicity to patients has been an ongoing topic of discussion, driving research into less cytotoxic CPAs. Cell membrane toxicity is the most common type of specific toxicity associated with DMSO.

Shu et al. also reviewed the effects of DMSO on a variety of organisms and biological systems [20]. However, DNA methylation and histone alterations have been reported to reduce survival and induce cell differentiation [21, 22].

1.1.3 Ethylene glycol (EG) and propylene glycol (PG)

Ethylene glycol (EG) is approximately half as permeable to human egg cells as propylene glycol (1,2-propanediol) (PG) or dimethyl sulfoxide (DMSO), thus increasing membrane damage through osmotic stress. However, EG is the cryoprotectant of choice because it is less toxic than other drugs [23].

It is important to note that using a relatively high concentration of EG (15%) was prepared in an equimolar mixture with DMSO. This suggests that post-vitrified blastocyst transfer has no negative impact on perinatal outcomes compared to post-vitrification post-transfer [24].

Most embryos are more permeable to PG than to glycerol. PG has few systemic toxic effects and is used safely in many foods. PG has been used as an antidote for EG poisoning [25]. Nevertheless, PG (1,2-propanediol) is often toxic when used as a CPA agent. For example, 1,2-propanediol above 2.5 M has been shown to impair the developmental potential of mouse zygotes by lowering the intracellular pH [26].

1.2 Non-permeable cryoprotectants

Impermeable cryoprotectants are a special class of high molecular weight, impermeable molecules. These include starches such as hydroxyethyl starch and polyethylene oxide, sugars such as trehalose and sucrose, and polyvinylpyrrolidone. They cannot enter cells and therefore remain extracellular during cryopreservation by exerting osmotic effects to support rapid cell dehydration, lower freezing temperature of the medium, and reduce extracellular ice formation [27, 28]. They are used to protect cells from rapid cooling [9–11].

Due to their low toxicity, they are commonly used as extracellular CPAs. Typically, these are not used alone, but together with permeable intracellular standard CPA [29]. However, glucose has specific toxicities such as binding to proteins [30] and causing glycation as a reducing sugar [31].

Many studies report that trehalose is superior to other sugars such as trehalose. In maintaining membrane stability, liposome stability is maintained during drying and preservation of biomaterials [32].

Sucrose is considered a cosmic [33]. Sucrose is used as an extracellular CPA for embryo and oocyte vitrification [34]. However, under acidic conditions, sucrose is more easily hydrolyzed to reducing sugar monosaccharides than the disaccharide trehalose [32].

2. Cryopreservation methods

2.1 Controlled slow-rate freezing

Slow freezing or slow programmable freezing technology was introduced in 1966 [35]. This cryopreservation technique was introduced in the 1980s. As the name suggests, it involves slow freezing of eggs or embryos. Treat the cells first with antifreeze (antifreeze) to protect them in the process. Then gradually cool; at a rate of 1–2°C per minute: from +24°C to −7°C, then sowing, 10 minutes after sowing the temperature drops to −30°C, and finally immersion to −196°C for storage of liquid nitrogen [36]. However, optimal cooling rates vary widely between cell and tissue types [37].

2.2 Vitrification

The word vitrification comes from the Latin vitrum, meaning glass [38]. Vitrification has replaced traditional slow freezing as the primary method for gamete and embryo cryopreservation, while reproductive cryopreservation is slowly shifting research focus to vitrification, a cheaper, faster, and simpler technique [39–41].

Vitrification differs from slow freezing in that it avoids the formation of ice crystals in the intracellular and extracellular spaces [38]. So many laboratories around the world have completely replaced slow freezing with vitrification in order to improve cryogenic survival outcomes [42, 43].

Vitrification is an alternative freezing method based on the solidification of solutions at low temperatures, not by ice crystallization, but by a large increase in viscosity during cooling [44]. It is achieved by briefly exposing the embryos to high concentrations of cryoprotectant (~7–8 M), followed by direct immersion of the embryos in liquid nitrogen, resulting in ultra-rapid cooling at approximately 20,000°C/min. With this technique, a glassy amorphous state can be achieved, and the formation of intracellular and extracellular ice crystals is prevented [45].

This technique can be used to freeze sperm, oocytes, fertilized oocyte (zygotic) embryos, umbilical cord blood, and reproductive tissue in testes or ovaries [46].

3. Fertility preservation interventions

Fertility preservation may be indicated for the following indications: Women diagnosed with cancer, women with a disease, surgery, or treatment that may affect future fertility (including lupus, endometriosis, and Turner syndrome) Fertility declines with age in women, transgender men, and women worried about aging.

3.1 Oocyte cryopreservation

Oocytes are cells about 120 microns in diameter with a thick membrane called the zona pellucida. Egg cells are often referred to as the largest cells in the human body.

Surface and volume play important roles in the outcome of cryobiological processes. Therefore, freezing and thawing of unfertilized oocytes require extensive empirical and theoretical knowledge [45, 47].

Oocyte cryopreservation has become an important method for preserving female fertility in medical and non-medical indications [48, 49].

For women with age-related selective fertility, without a male partner, or without donor sperm, oocyte cryopreservation may offer another experimental option under stringent institutional review board (IRB) protocols, early data show promising results [50].

Unfortunately, oocyte cryopreservation is technically more complex than embryo cryopreservation, and unfertilized oocytes are more susceptible to damage during cryopreservation, so these procedures may have lower rates of unsuccessful pregnancies [51]. Cryopreservation of unfertilized oocytes is more technically challenging than embryo cryopreservation but has less ethical and legal implications.

Cryopreservation of human oocytes can be performed by conventional slow freezing or vitrification [46, 52]. Cryopreservation of immature oocytes in prophase I (follicle stage) has been proposed as an alternative to standard oocyte cryopreservation, as these oocytes are thought to be less susceptible to cryo-damage due to the absence of a spindle and different Membrane permeability [53].

ICSI is recommended for insemination of frozen and thawed oocytes because this method offers a reasonable chance of fertilization compared to in vitro fertilization [54].

Chen in 1986 reported the first pregnancy resulting from the slow freezing and rapid thawing of human eggs using DMSO (dimethyl sulfoxide) as a cryoprotectant [55].

Van Uem reported the second litter after cryopreservation of oocytes using a cryopreservation technique different from that described by Chen [56]. Several pregnancies have been reported after oocytes were cryopreserved—thawed and received intracytoplasmic sperm injection (ICSI) [57].

Kuleshova announced the birth of the first child from vitrified oocytes. The newborns were normal and healthy [58]. Other authors successfully used vitrification and found another 10 pregnancies [59].

The total number of children born after fertilization of frozen and thawed oocytes worldwide exceeds 1500 [51, 60]. Furthermore, no intellectual and/or developmental deficits have been found in children born from frozen oocytes [54, 61, 62].

Slow freezing is one of the methods of cryopreservation of oocytes. Compared with the fresh cycle, it has some limitations, such as low oocyte survival [63–65], increased risk of oocyte senescence [63, 66, 67], and reduced embryonic development [63].

Cao et al. conducted a randomized study to compare survival, fertilization, early embryonic development, and meiotic spindles in slowly frozen and vitrified and thawed human oocytes (n = 605) Assembly and Chromosome Arrangement. The vitrification group had significantly higher oocyte survival rate, fertilized egg and developing embryo division rate, and blastocyst development percentage than the slow freezing group (91.8%, 78.0%, 24.0%, 12.0% vs. 61.0%), 54.4%, 42.3%, and 33.1%, respectively). They also noted that vitrification was superior to slow freezing methods, resulting in improved oocyte survival, fertilization, and embryonic development in vitro [68].

Konc et al. used Polscope to determine the presence, position, and spindle dynamics/displacement in each oocyte. They examined frozen and thawed human oocytes

before and after thawing and for 3 h in culture and found that the spindle did not always return to its original position within the oocyte [69].

After thawing and culturing, they were able to see spindles in 84.3% of the oocytes. However, vitrified oocytes tend to reassemble their spindles more efficiently and faster than slowly cooled oocytes [70]. Cobo et al. found comparable spindle recovery after vitrification and slow freezing for 3 hours [62].

Cobo et al. in an oocyte donation program published the results of a randomized controlled trial of more than 3000 fresh oocytes and 3000 vitrified oocytes (92.5% survival rate). Randomized controlled trials demonstrated no adverse effects of vitrification on subsequent fertilization, development, or implantation [71]. Nagy et al. have also reported similar results in an oocyte donation program and Herrero et al. use the same vitrification protocol [72, 73].

3.2 Pronuclear stage (2PN) cryopreservation

Until recently, our laboratory and others in Germany have focused on cryopreservation of embryos at the prokaryotic stage (PN). PN freezing was performed because of the reported clinical success rates and if embryo selection and thawing techniques improve over time, it ensures that patients have access to a larger cohort of potential embryos [74]. However, at the PN stage, there is evidence that cryopreserved embryos may suffer from damage to prokaryotic integrity and thus their developmental potential may be significantly impaired [75].

Veeck et al. to improve the overall pregnancy rate per search cycle [76], have described cryopreservation of excess prokaryotic stage embryos. They reported that if cryopreserved prokaryotic oocytes survive freezing, thawing has similar implantation and pregnancy potential compared to fresh conception. However, a limitation noted by these researchers is the low rate of embryo survival after thawing (68%) [76].

In another study of more than 300 single frozen embryo transfers of day 2 4-cell stage embryos and embryos that lost only one blastomere (25%), a similar transfer was performed on fully intact frozen embryos and an efficient operation, and fresh embryos were also obtained [77].

As a result, many centers have completely phased out the use of slow freezing and, after long-term adoption of traditional slow freezing, have been replaced by conventional vitrification procedures.

Schroeder et al. reported a pregnancy rate of 10.2% using slow cryopreservation of cryopreserved human fertilized eggs [78].

Sang Shan et al. compared slow freezing and vitrification methods in cryopreservation of 2PN zygotes and reported a 100% survival rate of 5881 vitrified zygotes using cryotop as a carrier [79].

Among 340 vitrified embryos, the zygotic PN stage after vitrification was reported to have a 100% survival rate, a high pregnancy rate (36.9%), and a low miscarriage rate (17.42%). In addition, vitrification of 2-PN fertilized eggs has a high pregnancy rate of 46.2% and a survival rate of 97% [80].

3.3 Embryo cryopreservation

Embryo freezing and thawing are considered to have a higher survival rate than oocyte cryopreservation. The first successful embryo cryopreservation was achieved when the research team froze mouse embryos in polyvinylpyrrolidone

(PVP) [81] and the earliest pregnancy in frozen-thawed human embryos was reported in 1983 [82].

Rall and Fahy successfully vitrified embryos using high concentrations of cryoprotectant (CPA) and relatively low cooling and heating rates [38].

Embryo cryopreservation is a critical procedure for embryo transfer (ET) discontinuation due to the risk of ovarian hyperstimulation, endometrial bleeding, elevated serum progesterone levels on the day of ejection, or other unexpected events. There is still much debate about optimal tiers, protocols/procedures, and the use of cryoprotective additives (CPA).

Successful pregnancies and live births by thawing frozen human embryos were first reported in the 1980s. Ferraretti et al. showed that the pregnancy rate (PR) and live birth rate (LBR) of patients who subsequently received cryopreserved embryo thaw were like those of patients who received fresh transfer [83].

The average potential of cryopreserved embryos to become live is about 4%, and babies born from cryopreserved embryos do not exceed 8–10% of the total number of babies born with AR [84].

The results of a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle between 1986 and 2007 showed that the length of storage, whether by in vitro fertilization or oocytes, had a significant effect on clinical pregnancy, miscarriage, implantation, or survival. Yield did not significantly affect the donation cycle [85].

Compared to traditional slow-freezing methods, embryo vitrification is a recently introduced ultra-rapid cryopreservation method that prevents freezing within the suspension, transforming it into a glass-like solid, avoiding damage to cells or tissues [86, 87].

Embryo vitrification (VT) was first clinically introduced in Australia in 2006 and is now used for nearly three-quarters of the autologous thaw cycles for transferring blastocysts [88, 89].

Vitrification has been reported to significantly improve pregnancy, delivery, and implantation rates compared to slow freezing of cleavage-stage embryos and blastocysts [90].

Sifer et al. presented the results of a prospective observational study (58 cycles) where early cleavage stage good quality embryos were vitrified and warmed with the results of a retrospective series (189 cycles) where embryos were thawed after a slow freezing procedure (SF). They concluded that the post-thaw survival of vitrified embryos was significantly better than those of embryos resulting from slow freezing procedure. Then, a better clinical pregnancy rate (CPR) per thawed embryo cycle was observed following vitrification [91].

Debrock et al. compared the live birth rate (LBR) per embryo (day 3 cleavage stage embryos) after freezing and thawing by slow freezing or vitrification. They showed that the survival rate after vitrification was significantly higher than that after slow freezing, and the LBR per embryo was significantly higher after vitrification (16%) than after slow freezing (6%) [92].

Zhu et al. compared a retrospective cohort study of 5613 infertile patients with 7862 frozen and thawed day 3 slow frozen (SF) embryos and 3845 vitrified and heated embryos. Day 3 embryos. The proportion of high-quality embryos after thawing in SF was lower than in VT. In a single frozen embryo transfer (FET) cycle, pregnancy and implantation rates were similar between the two groups (35.0 vs. 40.8% and 34.6 vs. 35.9%, respectively). Also, for dual FET, pregnancy rates per cycle were similar between groups (58.8% vs. 58.4%). The implantation rate per embryo transfer was

significantly higher in SF than in VT (38.8% vs. 34.6%). However, SF protocols for cryopreservation of day 3 embryos should be considered [93].

Pooled data from 7 randomized controlled trials (RCTs) (3615 embryos) showed a significant increase in cryopreservation of embryos after vitrification compared to slow freezing ($P < 0.001$) [94].

When embryos are placed in a freezing solution containing intracellular cryoprotectants (ethylene glycol, propylene glycol, glycerol, dimethyl sulfoxide (DMSO)), due to the extracellular concentration (osmolarity) of cryoprotectants from naive cells Gradient) is higher, intracellular water will leak from the cell. After reaching equilibrium, it gradually diffuses into the cell by cryoprotectant and shrinks until osmotic equilibrium is reached; the cell returns to its normal appearance [95, 96].

The main problem with using cryopreservation techniques is that embryos may be lost due to cryogenic injury. Possible risks of injury to cryopreserved and thawed embryos include exposure to biochemical intracellular ice formation (ICC), cytotoxicity of cryoprotectants due to hyperosmolarity, physical damage (zona pellucida), and deoxygenation during embryo handling ribonucleic acid (RNA) damage. During embryo storage [97].

The most important known mechanism of damage to the cells that occur during cooling to low sub-zero temperature includes chilling injury, ice crystal formation, and fracture damage. In controlled slow freezing, embryos are osmotically equilibrated by incubating in approximately 1–2 M permeable and impermeable CPA prior to freezing. This protects the embryo from the formation of intracellular ice crystals. The extracellular ice is then seeded to form, and the embryos are then cooled at a controlled rate to −30 to −70°C using a programmable slow-speed freezer at 0.2–2.0°C/minute (min). Finally, embryos are immersed in liquid nitrogen (LN2) for short- or long-term storage [98, 99].

The only danger to cryopreserved cells is suspected to be DNA damage caused by background radiation. Human gametes can safely withstand 3–4 G radiation. Thus, human cells can safely survive for hundreds of years at typical terrestrial background radiation levels of 0.1 cGy/year. However, cosmic rays may be less harmful to embryos stored in high-quality cryogenic tanks than previously thought [100].

Sang Shan et al. vitrified cleavage stage embryos with EG + DMSO+sucrose and showed a small but significant improvement in survival (98% vs. 91%), but no difference in pregnancy rates relative to slow cooling [79]. In a similar comparative study, slow cooling and vitrification were found to have no differences in survival and implantation rates [101].

Balaban et al. observed a higher survival rate (94.8% vs. 88.7%) and a higher rate of intact embryos (73.9% vs. 45.7%) in the vitrification group using the PG + EG + sucrose solution group compared to the slow solution group). Day 3 embryos were frozen in 1.5 MPG + 0.1 M sucrose [102].

3.4 Blastocyst cryopreservation

Cryopreservation of blastocysts is a challenging task due to the size of blastocysts and presence of blastocysts. Since blastocysts contain a lot of water, the formation of ice crystals may be a major factor affecting blastocyst survival. Cohen et al. reported the first infant born after frozen/thawed blastocyst transfer [103].

Cryopreservation at the blastocyst stage has mainly been performed using slow methods with acceptable results [104–106]. It has been suggested that vitrification results in less apoptosis in blastocysts compared to slow freezing [107].

Outcomes of blastocyst-stage vitrification have improved significantly since 2001 [108, 109], with survival rates as high as 100% [110, 111] and 53% pregnancy rates reported by various investigators [79, 110–112].

Several studies have reported increased blastocyst survival when vesicle volume is artificially reduced using glass microneedles [113], 29-gauge needles [86], and hand-drawn Pasteur pipettes for micropipettes [113, 114].

Liebermann and Tucker reported a survival rate of 80.6%. Therefore, highly reproducible vitrification using the Cryotop method is superior to slow freezing. Furthermore, to date, no other technique has consistently achieved the excellent results obtained using this method [115].

Kuwayama et al. found in a comparative study that vitrified blastocysts had a slightly higher survival rate (90%) than slowly cooled blastocysts (84%). However, pregnancy and live birth rates per transfer were not significantly different [79].

In a study of more than 500 blastocysts per group, Liebermann and Tucker found significant differences in survival (96.5% vs. 92.1%), pregnancy per transfer (46.1% vs. 42.9%) and implantation rate (30.6% vs. 28.9%) between the vitrified and slow freezing groups [116].

Loutradi et al. found that blastocyst survival after vitrification was significantly higher than after slow freezing (odds ratio [OR]: 2.20, 95% confidence interval [CI]: 1.53–3.16) [117]. In addition, Hong et al. found a high pregnancy rate (70.5%) and implantation rate (40.6%) when using the new vitrification technique [118].

Recent studies have reported similar clinical outcomes between vitrified blastocyst transfer and fresh blastocyst transfer cycles when similar quality blastocysts were transferred [93, 119].

Cobo et al. vitrified 6019 embryos with cryogenic glass and showed that 97.6% of embryos survived on day 6, compared to 95.7% on day 5, 94.9% on day 2, and 94.9% on day 3, 94.2% at 6 days [120].

3.5 Ovarian tissue cryopreservation

The ovary has hundreds of primordial follicles containing immature oocytes that are small, quiescent, less differentiated, and devoid of banded cells. Due to the lack of zona pellucida and cortical granules, this immature oocyte can tolerate cryopreservation [121].

Ovarian tissue cryopreservation (OTC) is an evolving technique, although limited outcome information is available. Ovarian tissue can be cryopreserved for later ovarian tissue transplantation in prepubertal patients or when immediate chemotherapy is required [122].

Ovarian tissue is collected laparoscopically and frozen and can later be thawed and reimplanted in situ (in the pelvis) or ectopic (into the subcutaneous tissue of the forearm or abdomen). The cryopreservation process of ovarian tissue involves freezing thin slices of ovarian cortex, which contain a rich reserve of primordial follicles. This method of investigating fertility preservation requires only ovarian cortical tissue [123].

The first frozen-thawed ovarian transplantation was reported in 2000, and since then, several successful pregnancies due to these procedures have been reported [124]. Studies have reported restoration of ovarian function using both approaches [125, 126].

The potential risk of cancer recurrence in preimplantation tissue not exposed to chemotherapy may limit its use in cancer patients, at least until in vitro maturation of immature oocytes becomes more standard [125, 127, 128].

The advantage of cryopreservation of ovarian tissue compared to mature oocytes is that primordial follicles in the ovarian cortex are more resistant to cryoinjury [129]. However, long-term studies have reported graft function for up to 11 years [127, 130].

Porcu et al. reported the first birth of healthy twins in a patient who underwent bilateral oophorectomy for ovarian cancer and was pregnant with her own cryopreserved oocytes [131]. Besides, 131 pregnancies and 75 live births (expected to exceed 200 by 2020) have been reported after slow freezing and transplantation, whereas only 4 deliveries have been described after vitrification [132].

In addition, many deliveries between identical twins using fresh ovarian transfer have been reported [133]. There are also reports of births from two sisters with HLA-compatible whole-fresh ovarian transplants [134].

There are two methods of OTC: slow freezing and vitrification. Early studies have shown that slowly frozen ovarian cortex preserves better than vitrified ovarian tissue [135]. Slow freezing has been the traditional technique for many years, despite reports of massive follicular pool loss and excessive stromal cell damage [136].

Xiao et al. reported that a new vitrification technique was comparable to slow freezing in preserving primordial follicles in human ovarian tissue. The proportion of morphologically prominent primordial follicles was significantly reduced by vitrification compared with slow freezing [93].

To date, only two live births following vitrification of ovarian tissue have been reported and all other live births were caused by slow freezing of the ovarian cortex [137, 138]. Twelve studies collected data on intact primordial follicles, and an overall pooled analysis showed no difference between vitrification and slow freezing for this endpoint [139].

However, it has recently been suggested that vitrification has beneficial effects on granulosa cells and ovarian stroma, providing equal or better results than slow cooling to protect ovarian tissue [139].

4. Storage in LN2 containers after slow freezing and vitrification

In the field of assisted reproductive technology, little is known about the risks of long-term storage of cryopreserved cells, because vitrification is the solidification of a liquid without forming a crystalline structure—a physically disorganized and therefore potentially unstable system. This raises the question, if this changes over time, does this significantly affect the cryosurvival and implantation potential of vitrified gametes and embryos?

Subsequently, the possible effects on neonatal health remain largely unknown. A study by Wirleiter et al. showed that storage of vitrified blastocysts under sterile conditions did not affect blastocyst viability. In addition, no significant differences were observed in survival rates after warming between the first year of storage and after 5–6 years of storage (83.0% vs. 83.1%); nor did the pregnancy rate decrease (40.0% vs. 38.5%). Furthermore, no increase in neonatal malformation rates was observed over time [140].

To date, there have been no reports of cross-contamination between germ cells and tissues stored in cryovials. Cobo et al. showed that viral sequences (HIV, HBV, and HCV) were not detected in liquid nitrogen samples from containers containing oocytes and embryos from chronically infected patients [120].

To date, neither open systems nor closed systems have resulted in disease transmission during vitrification. However, to ensure biosafety during cryopreservation, aseptic methods are recommended [43].

Germ cells and tissues must be cryopreserved and stored in accordance with European Organization Directive 2006/17/EC (European Union [EU] Directive 2006/17/EC) to prevent pathogen transmission or cross-contamination of samples. Patients must be screened for blood-borne viruses (BBV), such as HIV, Hepatitis B, and C, before processing and freezing gametes/embryos and storing germ cells and tissues for positive and negative patients, respectively. Periodic cleaning of storage containers is also considered good laboratory practice (GLP) for decontamination of viral and microbial agents [141].

5. Conclusion

Vitrification is now the method of choice for cryopreservation of oocytes due to better results than slow freezing, but more standardized applications are still needed. Transfers of fresh or cryopreserved embryos still performed statistically better than embryo transfers obtained from cryopreserved oocytes. Only a few centers with extensive experience in cryopreservation are comparable between frozen embryo transfer or oocyte cryopreservation embryo transfer.

Conflict of interest

The authors declare no conflicts of interest.

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Chapter 4

Ovarian Tissue Cryopreservation Guidelines

Mahboubeh Vatanparast

Abstract

With the increase in the survival rate of cancer patients, there has been a growing interest in the field of fertility preservation. One of the main methods in this aim is ovarian tissue cryopreservation, especially for prepubertal girls. From the early time of introducing this opportunity as a chance to preserve future fertility in cancer patients, following gonadotoxic treatments, many guidelines have been published, to introduce the real indications. The need for these guidelines seemed very urgent, and attracted great interest, because this method was performed as an experimental and no standard clinical option, for many years. So patient selection should have been done with the most standard and highly accurate criteria, which could analyze the cost/benefit of this technique after multidisciplinary evaluation, for each patient, individually. For many years the specialist believed that all caution must be taken in referring patients for this technology. To ensure that cancer patients receive high-quality uniform treatment, evidence-based clinical practice guidelines (CPGs) are needed. CPGs are essential to enhance care quality and decrease heterogeneity in practice and costs. The guidelines can provide clear advice on the best practice in the field of female FP, based on the best available evidence.

Keywords: ovarian tissue cryopreservation, gonadotoxic treatments, guidelines, female fertility preservation, premature ovarian insufficiency

1. Introduction

cancer survivors have increased dramatically with the improvements in cancer treatment [1]. However, cancer incidence, in the young age group, has increased, during the past 30 years, the mortality rates have declined and the 5-year survival rate has increased from less than 50% in the 1970s to 80% these days, and the 10-year survival rate is estimated 75% [2].

Cancer in adolescents and young adults (AYAs) occurs between 15 and 39 years old. Cancer-diagnosed AYAs are different from other age groups in many components, such as tumor etiology, biology, prognosis, intrinsic and extrinsic risk factors, cancer types and survivorship as well as the effectiveness of treatment. Besides, AYAs suffer more from long-term effects of treatment compared with those older ages, including sexual dysfunction, infertility, and future cancers [3].

Chemotherapy and radiotherapy may have gonadotoxic effects, and compromise ovarian function [4]. A positive correlation has been found between the risk of

gonadal harmful effects and the patient's age, which may be due to the previous reduction of the follicle numbers in older females. Additionally, the type of chemotherapy regimens and the dose are the other determinants of the degree of cytotoxicity [5, 6].

With the increase in the long-term survival rates of cancer patients who have experienced chemo/radiotherapy, there's been greater attention to the long-term effects of treatments, such as premature ovarian failure (POF) [6]. A mixture of health and quality-of-life problems have been created for the young survivors, many of which were not predicted at the time of cancer diagnosis [4].

It needed to be discussed with the patients the risks of cancer treatments on reproductive health, and the options for fertility preservation, before treatment. Consultation with a reproductive endocrinologist is of great value in providing adequate information regarding the side effect of cancer therapies on reproductive consequences and the chance of success rates for the various fertility preservation strategies. It was shown that almost 30% of cancer patients under 50 years old request more information about premature ovarian insufficiency or the probable risks to their children's health, and a third of them would have tended to have a fertility consultation before treatment started [7].

Gametes, embryo, and gonadal tissue cryopreserving may help to preserve fertility, and avoid damage to reproductive organs. Sperm and embryo freezing are well established (clinical stage), but oocytes and ovarian tissue freezing are still experimental [1].

However, in some parts, we referred to the other ways of fertility preservation (FP) options, but the main aim of this chapter is a review of the existing guidelines regarding ovarian cryopreservation, as a fertility preservation option. A comprehensive study of the available literature was conducted, to find updated and evidence-based guidelines and recommendations.

2. The emergence of oncofertility

Recently, with the increased survival rate of cancer patients, there have been much attention and interest in the long-term effects of treatment on future fertility [2].

Oncofertility is a domain that connects oncology with fertility and having a comprehensive view of these fields got it capable to introduce a standard of care in many institutions. This field has been developed simultaneously with the other life-preserving advancements in the oncology care unit, such as earlier diagnostics, targeted cancer therapies, new methods with less radiation dose, and local surgical procedures [8].

It passed more than half a century since the first time the concept of "ovarian tissue cryopreservation" was proposed, but it took 12 years, until achieving (reaching) the first successful human ovarian tissue cryopreservation, and the first live birth, following this technique has been reported in 2004. For many years, ovarian tissue cryopreservation and transplantation were done as experimental, in many fertility centers, until 2019, when this approach was accepted as a clinical technique, for fertility preservation [5].

This success has been achieved with the improvement in the technique, protocols, cryoprotectants agents, devices, as well the exact timing for equilibration, throughout many scientific studies, as it got qualified enough, to be considered as "the clinical approach" [9].

3. Female fertility preservation strategies

increasing the knowledge of the possibility of fertility loss following cancer treatment has led to huge growth in the fertility preservation field, throughout the last two decades. ESHRE 2020, described fully these options, based on the individual case [10].

There are some options for female fertility preservation before cancer treatment. For female fertility preservation, the most successful standard method is emergency IVF and embryo cryopreservation, as an established part of assisted reproduction, before cancer therapy [7, 10].

However, this method is not suitable for pre-pubertal girls, and young patients when there is no partner or when there is not enough time to delay cancer treatment. Oocyte and ovarian tissue cryopreservation are the other less effective options that are still experimental [10, 11].

Ovarian transposition or fertility-sparing cancer surgery is the other option that helps minimize the destructive effects of cancer treatments [10, 11]. Besides restoration of fertility, ovarian tissue transplantation in the POI can restore endocrine and hormonal function [12], also it can be done at any time during the menstrual cycle [13].

In addition, GnRH agonist co-administration also may provide some fertility protection against gonadotoxicity of the chemotherapy, but still prospective controlled trials are needed to approve this method as an established clinical method [7, 10]. In vitro oocyte maturation (IVM), and oocyte cryopreservation are the other choice methods, especially for women with age-related fertility loss, besides women who seek FP for medical indications [10].

Despite the introduction of these strategies, FP stays a particular challenge in the most common candidates such as hematological cancers (leukemia, Hodgkin's lymphoma, and non-Hodgkin's lymphoma) and breast cancer [14]. The most challenge is the patient's selection criteria, under 35 years old, when the ovarian reserve is still high, a realistic chance of 5 years survival rate, and when there the risk of premature ovarian insufficiency is at least 50% [15].

4. Ovarian tissue cryopreservation as an experimental method for FP

Nearly 20 years passed since the first human pregnancy report following ovarian tissue cryopreservation (OTC) [16], but until recently this method was being considered an experimental method. Embryo and gametes (sperm, and oocyte) cryopreservation are done as the standard practice worldwide, but OTC is a newly established method, which harbors many challenges. One of the reasons is that OT is a complex of various cell types with different physical structures and water permeability, that need a different process to survive during freezing [17].

The other challenge is the methods of cryopreservation, vitrification vs. slow freezing. There are two main methods for OTC; slow freezing (SF) and vitrification (VF). The conventional method is slow freezing, which is the base protocol for OTC worldwide, and nearly all live births have been reported to follow this method. The other is vitrification which compared to slow freezing is a new approach, with small numbers of live births. Some advantages and disadvantages have been reported regarding these methods [18].

The base for slow freezing is tissue exposure to the cryoprotectant agents (CPA), cooling of the tissue to a special temperature using programmable freezers, and final liquid nitrogen (LN_2) immersing (−196°C). DMSO (1.5 M) has been used as CPA in this method [19].

Vitrification protocol is done commonly using equilibration and vitrification solution exposure (increasing concentration of CPAs), and final LN_2 immersion. Ethylene glycol (EG) and dimethyl sulphoxide (DMSO) can be used as the CPAs in this method (7.5% for ES, and 20% for VS) [20].

However, SF has been used as the standard method, in many centers, but some properties of this method created an interest in vitrification. Besides this method is time-consuming, because of the need for a controlled-rate cooling device which is expensive. Also, the probability of cryoinjury is high in the formation of the intracellular ice crystal. Then vitrification was introduced for this purpose, instead of slow-freezing, because of time-saving, and rapid cooling with no need for expensive equipment [17]. Much research has been done to introduce the best method, but the results are controversial [21], but among numerous studies, rigorous documents have been earned that support VF as an alternative way to the SF method [22–28].

The other issue is the techniques of ovarian cryopreservation; ovarian cortical fragments or whole ovary.

Since most primordial follicles are located within the ovarian cortex, so cryopreservation of the small part of cortical tissue enables the storage of large numbers of oocytes. It mainly is recommended to obtain ovarian tissue before cancer treatment. For this purpose, ovarian tissue is commonly obtained by the laparoscopic approach, also it can be done by mini-laparotomy or during ovarian transposition [29].

For the fragment cryopreservation, the cortical tissue will be transferred to the lab, then thin cortical strips will be prepared, using a scalpel, the optimal size will be $\sim 5 \times 10 \times 1\ mm^3$ thick, before freezing [18, 20, 30].

The whole ovarian cryopreservation consisted of ovarian removal with the vascular pedicle, through laparotomy or a laparoscopic approach. One of the big advantages is vascular anastomosis of the thawed ovary, which probably provides a larger follicular reserve and also a longer ovarian life span. But there are no currently rigorous documents to support this hypothesis, mainly because of the created damages by applying freezing procedures for a big sample as a human ovary [31].

In one study on sheep ovary, it was shown that the directional freezing method enhances the ovarian tissue cryopreserved viability, for both whole ovary, and ovarian fragments [31]. The physical concept for directional freezing is a sequence of four heat-conductive units positioned in a line. The blocks are set at different temperatures, which creates a temperature gradient. Freezing tubes will be passed, at 0.01 mm/s speed, along the thermal gradient, starting at +4°, and decreasing to −70° C. This results in a cooling rate of 0.3°C/min, and at the end, samples will be plunged into liquid nitrogen [32, 33].

The other problem is how to use cryopreserved ovarian tissue. However the OTC is now a relatively well-established procedure, but the restoration of fertility using the cryopreserved tissue remains a challenge. The only approach for the restoration of fertility is the re-implantation of ovarian tissue [34]. But the re-implantation process is accompanied by attrition of the non-growing follicles population, something about three-quarters [35]. Now the thawed ovarian tissue transplantation can be done in two ways; orthotopic or heterotopic. The other alternative approaches, such as *in vitro* follicular culture need additional research before accepting as an established practice for humans [34].

until 2017, worldwide over 130 babies have been born after ovarian tissue cryopreservation (OTC), and ovarian tissue transplantation (OTT). It was estimated that OTC resulted in 93% restoration of endocrine function [36], 29% successful pregnancy rates and 23% live birth rates [37].

One another important issue regarding OTC is how long the transplanted tissue will remain functional. However, there are some variations in the ovarian grafts' lifespan after ovarian transplantation, it seems that the endocrine function duration is longer than what is expected. On average, the follicular density of nearly 4–5 years will be preserved well, after transplantation, but up to 7–10 years of ovarian function duration also has been reported [36, 38].

For the OTS two surgical processes are needed; one for the extraction and the other for re-implantation [39]. To date, no standard operative technique has been established for obtaining ovarian cortical tissue. Few publications have dealt with the operative technique and outcomes, and the mentioned methods were as follows; ovarian cortical biopsy, unilateral hemi-oophorectomy, as well bilateral hemi-oophorectomy, and unilateral salpingo-oophorectomy [40].

All of the mentioned challenges, together, cause us to consider the OTC as an experimental method for female fertility preservation. Besides, there are some information gaps, which need to be addressed.

5. Whom, what, when, which, who information gaps

After the creation of the new approach (fertility preservation), some important questions arose, but there were no unique answers for them. One of them was; for Whom must it be proposed? What were the indications? When must it be done? Which strategy is useful, for each case? Who must provide fertility preservation consultation?

There are some studies, which tried to answer these questions, and some indications have been introduced, a range from very commonly known cases such as cancer patients [41], the latter as the social reason for pregnancy delay [42, 43], and age-related fertility loss [10], to the new indications such as transgender (fertility preservation for trans men) [43, 44], or Endometriosis [45]. However many guidelines suggested that fertility preservation consultation should be done before treatment is a great information lack in this area [46].

5.1 The challenge of "for whom?"

Ovaries or testes will not be affected in many cases, by the cancer treatment and physicians can reassure the patients. But there are also other cases, in which, the impact of treatment on fertility is unknown due to a lack of valid studies. Also sometimes the problem is the medical oncologists when there is a belief that there is no good choice/or chance, so discussing it will not help.

The related risk for premature ovarian insufficiency (POI), after chemotherapy, is influenced by some factors such as age, body mass index (BMI), type of treatment, and duration. For example, in breast cancer, the risk of amenorrhoea, after six cycles of CMF (cyclophosphamide, methotrexate, fluorouracil) was estimated at 33% for patients below 40 years and, 81% for patients ≥40 years old, also the risk for amenorrhoea was estimated 51.4% in women under 30 years old and 95.0% for the women above 30 years, after 8 sessions for dose-escalated of BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) [47].

5.2 The challenge of "which strategy?"

Which option is appropriate for each patient is the other gap, which must be exactly described in the guidelines. Some important criteria must be fulfilled in the

aim to classify a new technology and treatment as an established technique. They are efficacy, safety, procedure, and effectiveness to assess [48]. The ovarian tissue cryopreservation, as a new field of oncofertility, at first must be set up on nonhuman primate models and then would become appropriate for humans, the necessary step to move from the bench to the bedside [4].

Ovarian tissue cryopreservation is done by obtaining ovarian cortical tissue, the part that is rich in primordial follicles, before gonadotoxic treatment, by laparoscopy or laparotomy. Small fragments are prepared and then cryopreserved by the slow freezing or vitrification technique. After cancer treatment, the tissue will be transplanted in two ways of orthoptic (the pelvis) or heterotropic (outside the pelvis-abdominal wall, and forearm) transplantation. Orthotopic transplantation the most successful method has been accompanied by spontaneous pregnancies, but in heterotopic transplantation, IVF is necessary [46].

Because of the nature of being experimental, it is needed to identify the accurate indication for this technique [15].

In this regard, there are some guidelines by the American Society for Reproductive Medicine (ASRM) and American Society of Clinical Oncologists (ASCO) which recommend cancer patients must be referred for fertility preservation counseling, related to the risk of infertility after cancer treatment and must be aware of the appropriate plans [49–51]. But, there is not, always, appropriate consultation regarding the potential side effects of cancer treatment on future fertility, and also fewer patients are aware of the fertility preservation program [52].

The other big gap is the information delivery, while the medical oncologists are not exactly aware of the effect of their treatments on reproductive health and outcomes, clinical cancer patients are not routinely treated by reproductive endocrinologists. Some physicians also believe that it involves ethical issues to enable a patient to bear a child while the parent has a lowered life span or cannot take care of a child [1].

5.3 The challenge of "when"?

when must ovarian tissue cryopreservation be done? It was mentioned that over 90% of patients undergoing high-dose chemotherapy may suffer from ovarian failure [53] and cryopreservation should always be offered when the risk of POF is high (>30–50%) and the risk of ovarian metastasis is low [54]. It suggested that the best time is before the patient receives any chemotherapy [2, 15, 53].

Among many of the guidelines, ASCO mentioned individual cytotoxic treatment regimens and their effects on fertility. The guidelines mentioned that counsel the patient that this method is experimental [50, 55].

The patients' age was one of the most challenges which have been discussed in the various version. Some believed to refer all the patients of reproductive age [50], but some others considered age limitation, and recommended this to special ages, E.g. below 35, 37, or < 42 [51, 56, 57]. The reason for the age limitation is confirmation of the appropriate ovarian reserve [57].

A framework of guidance is needed for healthcare professionals to provide evidence-based care to the women and girls who are a candidate for fertility preservation [43].

5.4 The challenge of "who"?

Who must provide the fertility preservation consultation? The oncologists and hematologists may be the first ones, in the health care provider team, who find the

need for fertility preservation. After referral, the other healthcare professionals which have an important role as the oncologists, in the management of women with cancer, are obstetrics-gynecologists, endocrinologists, andrologists, nurses, embryologists, reproductive biologists, reproductive medicine specialists, psychologists, counselors, and general practitioners [8, 43, 49].

6. Fertility preservation guidelines

Fertility preservation, nowadays, is a necessary professional domain. It needs close coordination between teamwork which includes oncologists, reproductive biologists, and reproductive medicine specialists in various fields [8]. Many guidelines had been provided to describe the situations, in which fertility preservation is needed to be considered with the patients. However, there is still no general agreement between different guidelines in introducing criteria for ovarian tissue cryopreservation, such as patients' age limits or having a history of chemotherapy.

since the first time scientists succeeded in mammalian ovarian tissue cryopreservation, in 1994 [58], new indications have been introduced and the circumstances have been determined. It had been tried to introduce true indication, in each version. There are many heterogeneities in the criteria for introducing real candidates, such as patients' age limits, excluding patients with a history of chemotherapy, or specific diagnoses which preclude re-transplantation. Tissue preparation and freezing/thawed procedure are the other diverse worldwide [59].

To ensure that cancer patients receive high-quality uniform treatment, evidence-based clinical practice guidelines (CPGs) are needed. CPGs are essential to enhance care quality and decrease heterogeneity in practice and costs [52]. The guidelines can provide clear advice on the best practice in the field of female FP, based on the best available evidence [60]. Several guidelines have been published worldwide. Some of the guidelines focused on efficacy, and safety, while others have paid attention to the aspects of feasibility, and acceptability, and few had ethical considerations. A comprehensive guideline that encloses all aspects of FP, from patients' consultation to the outcome of techniques, would additionally help clinicians in this field.

These guidelines could introduce the potential candidates for OTC, when a woman is at risk for iatrogenic infertility, due to medical or surgical cancer treatment, for a benign or malignant condition [51].

Guidelines are set to address oncologists, gynecologists hematologists, endocrinologists, as well other healthcare providers, such as nurses, counselors, psychologists, and general practitioners who have a role in giving fertility preservation to cancer patients.

6.1 Edinburgh guideline (2005)

Edinburgh Criteria is one of them that was firstly published in 1996, and then slightly revised in 2000 [15]. Edinburg criteria once again and after multidisciplinary discussion and the report from the Royal College of Obstetricians and Gynecologists working group, described some criteria especially age limit) for patient selection, this guidance is applicable in a patient-specific manner and must be updated by the emerging new evidence and experience [61]. Besides introducing the patient selection criteria, two issues have been addressed; the probability of the congenitofabnormalities in children following chemotherapy, and the legal and ethical issues.

Regarding the concern for child abnormalities, the guideline reassures that a large study did not show any link between these. The guideline mentioned that this technique (FP) raised some critical ethical and legal issues, which must be considered before use. The costs/benefits of any intervention or decision must be evaluated, and all the advantages and disadvantages, in the short, and long term, must be considered. These proposed opportunities should not create unrealistic expectations, and should not bring adverse effects for the subsequent offspring.

Valid informed consent from the patients must be earned voluntarily, and from a competent person. Legal competence was discussed in this guideline, and also described that obtaining valid consent becomes more complicated according to the patient's age and their level of understanding of the discussed issues [61].

Edinburgh criteria validation for ovarian tissue cryopreservation had been done, in 2014, for young women and girls (younger than 18 years). The results validate the guidelines criteria for patient selection for ovarian tissue cryopreservation and show it can identify accurately the girls and young women at risk for premature ovarian failure [15].

Summarized recommendations of the Edinburgh Criteria for Ovarian Tissue Cryopreservation (2005, 2014):

1. Age < 30 (in 2005), Age <35 (in 2014)

2. Having no history of previous chemotherapy or radiotherapy (if the patients bellow15 years old, previous mild and low-risk chemotherapy can be considered)

3. A chance of long-term survival for 5 year

4. High-risk estimation for premature ovarian failure (>50%)

5. Informed consent from the patients or the parents (in children)

6. Negative syphilis, HIV, and hepatitis serology

7. No pregnant or existing children

But, the later study, again challenged one of Edinburgh's criteria, having no history of previous chemotherapy or radiotherapy, while Vatanparast and her colleagues 2021, presented a young girl with acute lymphocytic leukemia, which had a history of chemotherapy (15 sessions, with 30 mg vincristine and 975 mg Adriamycin) before referring. An anti-Mullerian hormone (AMH) had been requested, to survey the patient's fertility situation. It showed a premenopausal situation when reported within the normal range (3.66 ng/mL). after ovarian biopsy, the histology survey also showed a normal follicular density [62]. In the later guidelines, in 2022, it also was described that the OTC can be done also when the gonadotoxic risk is very high, but before the patients undergo chemotherapy is the ideal situation [39].

However, many studies have addressed fertility preservation before starting chemotherapy, fewer paid attention to the survey of pre-menopausal ovarian function [1, 50, 56, 57]. This gap in the available published guidelines is fully answered in the ESHRE guideline, 2020 [10]. There are some hormonal markers as well as ultrasound parameters that can estimate the ovarian reserve [63], behind the age or a history of chemotherapy! Unfortunately, ovarian biopsy only can be done through surgery or

laparoscopy, to obtain ovarian reserve from the histological survey. But, one of the most common indirect signs of ovarian reserve is hormone assay; Anti-Mullerian hormone (AMH). In the studies, AMH is accepted as a marker of ovarian reserve [64]. As it has been mentioned that a history of cancer alone does not provide enough documents to decide on patient sterility [7], and does not exclude the patient from the fertility preservation program. It seems that a big gap in the guidelines is the confirmation of pre-menopausal ovarian function, both before or after starting treatment. Also for the aim of ovarian reserve prediction, the other well-validated biomarkers, such as; FSH, LH, estradiol, Inhibin-B levels, ovarian volume, and the antral follicle count (AFC, by transvaginal ultrasound) also may be helpful. Serum AMH is reliable for the assessment after the age of 5, in addition, in older children, ovarian volume and antral follicle assessment can be recommended [65].

It's believed that small ovarian volumes, maybe a more acute marker in the aim of ovarian reserve estimation compared to abnormal hormonal concentrations,18 of which are indirect. Besides AFC, which shows the size of the existing primordial follicle pool may be a more sensitive marker of the ovarian reserve in comparison to ovarian volume. AMH which is produced by the growing follicles may be the most valuable representation of ovarian reserve [6].

6.2 American Society of Clinical Oncology (ASCO) guideline

The other guidelines also were prepared by the American Society of Clinical Oncology (ASCO) [49] and the American Society of Reproductive Medicine (ASRM) [1].

The ASCO guideline which was published in 2006, has been updated periodically. It was recommended that oncologists should consult with the patients regarding the possibility of infertility through their cancer treatment, before cancer therapy, and be aware of possible fertility preservation options to refer the patients to reproductive specialists.

Sperm and embryo cryopreservation which were introduced as the standard practices are widely available and the other options which were considered investigational should be performed in specialist centers [49]. This version was updated in 2013 [50]. In the new version, it was stressed that health care providers advise the patients regarding the potential effects of treatment on their fertility and inform them about a wide range of fertility preservation options, as soon as possible, during their treatment, although the patients initially focus on their cancer diagnosis. In this version, oocyte cryopreservation is mentioned as a standard practice. In this version, ovarian tissue cryopreservation is still considered experimental and not an established technique [50].

Summarized recommendations of the ASCO (2013):

1. Inform the patients of the individual risk of infertility and discuss the fertility preservation options with all patients of the reproductive age.

2. Refer both patients who intend to fertility preservation and are ambivalent to reproductive specialists.

3. Refer them for fertility preservation before treatment starts, as early as possible.

4. Fertility preservation consulting must be documented in the medical record.

5. Discuss common concerns

    - available fertility preservation (standard and experimental methods)
    - refer to consultation with an appropriate reproductive specialist, if the patients would like to learn more
    - discuss the possible impact of fertility preservation on the cancer treatment

6. Time:

    - before cancer treatment
    - sperm cryobanking is available for male cancer patients and can be repeated every 24 hours until collecting the necessary number of samples
    - for female patients, a period between 2 and 4 weeks may be needed for clinical techniques, or another experimental approach, so time is important for referring to a reproductive specialist.

7. Costs:

    - insurance coverage and list the benefits for the patients
    - some organizations may provide cost-saving programs

8. Risk estimated for future pregnancy and children after cancer treatment

9. Consultation with appropriate specialists:

    - reproductive specialists
    - mental health professionals
    - advocacy organizations.

6.3 American Society for Reproductive Medicine Clinical Practice Guideline

In ASRM (ethic committee) recommendations (2005), also as the other guidelines, consulting the patients regarding fertility preservation has been considered as the patient's right. This guideline, in a new approach, pertained to ethical and legal issues, in detail, for physicians and fertility specialists, as well cancer patients and offspring welfare, and the other issues are experimental vs. established options, the minor children's ability to give consent, the welfare of probably resulting children, and posthumous issues. This guideline described that concerns about the welfare of future offspring should not impede cancer patients from fertility preservation programs, also this guideline emphasizes the need for precise instructions in the case of a patient's death, unavailability, or other contingency after doing fertility preservation (disposition of cryopreserved gametes, embryos, or tissues). The necessity for

Preimplantation genetic diagnosis to prevent the birth of children with a high risk of hereditary cancer is ethically accepted [1].

The Practice Committee of ASRM, in 2014, after searching the existing literature, to evaluate the efficacy and safety of ovarian tissue cryopreservation, published new recommendations [29]. In this version, the indication for OTC has been described in detail, and the technique (cortical strips and whole ovary), methods of cryopreservation (slow freezing and vitrification), and transplantation have been discussed. In this guideline slow freezing has been reported as standard protocol. One of the concepts which were discussed in this version is safety concerns regarding the risk of reimplantation of cancer after transplantation. They suggest an alternative way for transplantation when there is a risk of reintroducing; if it is possible to isolate and recover immature oocytes from the ovarian cortex and use them for subsequent IVF, after doing maturation in vitro (either mature oocytes or embryos cryopreservation).

The guideline described the circumstances which must be realized until an experimental procedure is considered an established medical practice. The evidence published in the medical regarding their risks, overall safety, benefits, and efficacy must be documented from the only valid studies which were appropriately designed and performed by several independent researchers [29].

Summarized recommendations of ASRM (2005)

1. Cancer patients must be informed by their physicians about fertility preservation options before treatment.

2. Sperm cryopreservation and embryo cryopreservation are established methods.

3. Oocyte or ovarian tissue cryopreservation are experimental methods and should be offered only in a research setting.

4. The issue of the welfare of the offspring should not deny the patients receiving fertility preservation.

5. Parents may decide the preserve fertility for their minors.

6. Need for precise instructions about the disposition of the cryopreserved gametes, tissue, embryo, or oocytes of the posthumous.

7. The necessity of preimplantation genetic diagnosis to avoid the risk of inherited cancer.

Summarized recommendations of ASRM (2014)

1. Ovarian tissue cryopreservation and transplantation must be considered as an experimental option.

2. OTC is offered when immediate gonadotoxic treatment is required and for prepubertal girls.

3. OTC should not be done in benign conditions or for delaying childbearing.

4. The options for humans' OTC are cortical strips or a whole ovary.

5. Ovarian tissue transplantation sites may be done in both orthotopic and heterotopic.

6. Pregnancies and live births have been reported only after orthotopic.

7. No pregnancies have been reported from the heterotopic site or whole ovary transplantation.

8. One of the concerns of ovarian tissue transplantation is the potential risk of reintroducing malignancy.

6.4 Backhus et al. guideline (2007)

A guideline has been written by Backhus et al. [51] that was retrieved from the ASCO and ASRM's criteria. This version is wider than Edinburg's.

These guidelines help to identify correct candidates for OTC, when a woman with a benign or malignant condition is at risk for fertility loss, following medical or surgical treatment. In this guideline, also three methods of ovarian cryopreservation and transplantation, and follicles in vitro maturation were considered experimental and should not be recommended currently for patients who are candidates for fertility preservation, whenever there is no immediate or iatrogenic threat [51].

Summarized recommendations of Backhus et al. criteria [51]**.**

1. Age < 42 years

2. IVF cycle cannot be done, regardless of the presence of the partner

3. The pre-menopausal status is demonstrated or assumed

4. There is a significant risk for the acceleration of ovarian function loss

5. Informed consent must be earned from the adult patient

6. Informed assent is needed when the patient is below 18 years, besides informed consent from parental/guardian

7. patient can undergo an elective surgical operation

8. The patient plans to have a child in the future

9. when ovarian stimulation for oocyte retrieval is contraindicated, such as hormone-sensitive tumors.

6.5 Ferti-PROTEKT network[1] (2011–2019)

FertiPROTEKT network which first was established in 2006, encompasses the university-based, hospital-based, and private infertility and oncology centers. The main

[1] A specialized network and society (of physicians and biologists) in field of fertility preservation, in Germany, Austria and parts of Switzerland, www.fertiprotekt.eu

aim was to introduce local fertility preservation programs in Germany, Switzerland, and Austria, the techniques which enhanced the chance of achieving a pregnancy [57, 66].

The main theory of the formation of this network was that "a close coordination is needed between oncologists and reproductive medicine specialists, and reproductive biologists", so fertility-preservation activities should be organized, in a network structure, both as the medical-logistic network and as a professional medical society [8]. The three major networks introduced were as follows: (1) the Danish Network,[2] (2) The German-Austrian-Swiss network FertiPROTEKT,[3] and (3) The Oncofertility Consortium.[4] As these networks' structures are different in goals and logistics, so all the aspects of possible network structures are covered [8]. For example, the Danish network, gives practical implementation in the field of fertility-preserving techniques such as ovarian tissue cryopreservation and transplantation, in a small country. The second, the German-Austrian-Swiss network (FertiPROTEKT) organizes these (-fertility-protective techniques) in a large country, and the Oncofertility Consortium facilities knowledge transfer among its members.

In an attempt to introduce indications, in 2011, the Ferti-PROTEKT network prepared a practically oriented recommendation [57], and it was updated in 2018 [47]. This new version has a process-oriented approach, which stressed three main topics; disease prognosis, disease-specific treatment and risks of infertility, and disease-specific cryopreservation measures. Besides the risk for ovarian metastasis is also assessed which with the other mentioned topics are critical in deciding if fertility preservation is needed, or not. It could provide a disease-specific recommendation for fertility preservation measures [47].

Summarized some of the recommendations of the Ferti-PROTEKT network (2011):

1. In the case of breast cancers, fertility preservation must be considered especially in low-stage of cancer, in women below 35 years old, and in Hodgkin's lymphoma when the women's age is <40 years and the risk for POI is high.

2. In the case of Hodgkin's lymphoma, the chemotherapy regimens have a big role in decision-making as they may have very low to very high gonadotoxicity.

3. In the case of borderline ovarian tumors, fertility preservation usually is done through fertility-sparing surgery. In some cases, ovarian stimulation may be useful.

4. In some other cancer patients, a set of several factors must be considered, since there is a need to make an individual, stage-dependent decision. Some clinical and key recommendations have been provided by the guideline in the case of rheumatic and autoimmune diseases, cervical cancer, colorectal carcinoma, and other malignancies such as Ewing sarcoma, endometrial cancer, Borderline ovarian tumors (BOT), non-Hodgkin lymphoma, leukemia, etc.

[2] www.rigshospitalet.dk

[3] www.fertiprotekt.com

[4] www.oncofertility

5. In acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) ovarian tissue cryopreservation was considered experimental because of the high-risk estimation for ovarian contamination with the malignant cells.

At last, they concluded that answering the challenge of fertility preservation necessity depends on several factors such as prognosis, the risks for fertility loss, and some individual factors such as future family planning.

6.6 Cancer Council Australia (2014)

This guidance which pertained to many aspects of FP in adolescents and young adults (AYA), in detail, has been published by the Clinical Oncology Society of Australia (COSA), assists health professionals by introducing evidence-based recommendations and 'good practice points', to provide an effective consultation for AYA patients and their families.

Three main objects are as follows:

- the potential risk of infertility following cancer treatments
- fertility preservation strategies for patients, diagnosed with cancer
- list of potential late effects and the need for subsequent reproductive, sexual and endocrine health follow-up after cancer treatment.

Summarized recommendations of the Cancer Council Australia (2014):

1. Discussing fertility with the cancer patients
2. Supervising the fertility preservation strategy
3. informed the patients regard to the potential effect of cancer treatment on their fertility and reproductive health
4. Discussing the fertility preservation options
5. The need for long-term follow-up of their reproductive health, endocrine, and sexual function.

Regarding OTC, the guidance considered this technique as an investigational technique, which is appropriate for young women who are at high risk for ovarian failure, or when the other options are not suitable. Consult the patient that this technique is not a routine clinical practice. The ovarian tissue must be monitored for the presence of malignant cells. The guidance recommended managing the pregnancy after cancer treatment as a high-risk pregnancy, preferably in a tertiary center.

The guide also has a recommendation for infertile AYA cancer survivors. They should bedsides providing infertility counseling, and be informed about the assisted reproduction technologies opportunities, such as sperm, egg, or embryo donation, surrogacy, and adoption.

The complete version of this guideline is available via: https://wiki.cancer.org.au/australia/COSA:AYA_cancer_fertility_preservation [67].

6.7 Chinese Society of Gynecological Endocrinology affiliated to the International Society of Gynecological Endocrinology Guideline for Ovarian Tissue Cryopreservation and Transplantation (CSGE-ISGE) (2018)

Besides some guidelines which have no special target, a guideline has been published by the CSGE-ISGE to introduce the standard application of OCT in China. Based on Chinese specific conditions and after assessing the international guideline, they formulated the selection criteria as follows.

1. Age preferred ≤35 years old, for the assurance of good ovarian reserve, but if the ovarian reserve is confirmed and the patient asks for it, age limitation can be changed.

2. excluded the malignant ovarian tumors or when there is a high risk of ovarian metastasis.

3. good prognosis.

4. when the risk of POI is high because of the primary disease or the treatment.

5. laparoscopic or open abdominal biopsy is not contraindicated.

6. Radiotherapy and chemotherapy can be delayed at least 3 days.

7. Informed consent (the patients or their parents or guardian).

In this guideline, the authors described the good indication as the patients with tumors and nonmalignant diseases, as the aim of OTC is to preserve fertility and ovarian endocrine function. This method is suitable for prepubertal girls, patients who do have not enough time to postpone chemo- or radiation therapy, and women with hormone-sensitive tumors [45].

This guideline described that there is no uniform standard protocol for the timing of transplantation. It depends on the primary disease and can be done when the disease is cured and clinical rehabilitation happens. After full situation consideration, the patient's specific treatment is done.

commonly when the primary condition is cured, and the symptoms of menopause have been revealed due to ovarian function destruction; such as hot flashes and sweating, serum level of follicle-stimulating hormone (FSH) ≥ 25 IU/L, and anti-Mullerian hormone (AMH) <1.1 ng/ml ovarian retransplantation can be done at least 3–6 months after stopping chemotherapy [45].

Every month follow-up is needed to assurance of ovarian function recovery (reproductive and endocrine). After recovery, follow-up can be continued every 3–6 months.

For leukemia cases, there is no ideal fertility preservation program and OTC should be done before hematopoietic stem cell transplantation. Due to the high risk of reintroduction of malignant cells, ovarian transplantation should be performed with caution. This guideline also paid attention to ovarian endometrioma fertility preservation, while the inappropriate endometrioma removal can cause destruction effects on the ovarian reserve, and therefore assessment of ovarian function should be done before endometriosis surgery, since fertility preservation may be needed during the operation.

6.8 British fertility society policy and practice guideline (2018)

This guideline brought together the evidence literature for fertility preservation techniques and their outcome, as well as the associated risks, for medical reasons; both oncological and non-oncological cases. The guideline recommended consultation with women and girls about the risk of cancer treatment on their fertility and available preservation techniques. The four measures were: embryo, oocyte, and ovarian tissue cryopreservation, GnRH agonist administration, and ovarian transposition. In this version also ovarian tissue cryopreservation was still considered experimental. For benign and malignant conditions, current treatment modalities, which were accompanied by a better fertility-sparing profile, were described.

The guideline highlighted the role of psychological support in decision-making. The guideline also highlights that it is needed for the patients to meet the exact criteria to undergo invasive procedures, and FP must be done for curative intent [43].

The summary of its recommendations is as follows:

1. Reassure the patients that cancer treatment does not increase the risk of congenital anomalies or genetic disease

2. Radiotherapy to the uterus is probably with obstetric risks.

3. Inform cancer patients that the available data showed pregnancy does not increase the risk of cancer resuming, in many cancers.

4. The risk estimated for infertility, and premature ovarian failure should be done based on age, type of regimens, and dose of chemotherapy.

5. The risk of infertility, following pelvic, abdominopelvic, or craniospinal radiation depends on the field of exposure.

6. Inform the patients with breast cancer that they may suffer from delays in conception because of lengthy endocrine therapy, even if there is no need for gonadotoxic treatment.

Offer fertility preservation if:

1. There is a serious risk of infertility following the current cancer treatment.

2. There is a good chance for long-term survival.

3. The patient's situation permits ovarian stimulation and oocyte collection and does not jeopardize prognosis.

4. Cryopreserved embryos and oocytes have the success rates as the fresh ones.

5. To shorten the treatment duration and prevent the risk of ovarian hyperstimulation syndrome (OHSS) antagonist protocols are usually recommended. An agonist trigger reduced the risk of OHSS.

6. An anti-estrogen [letrozole, clomifene, or tamoxifen] prescription during ovarian stimulation in the case of estrogen-sensitive tumors.

7. Patients should be aware of the time limitation for storing their oocytes/embryos.

8. Available evidence shows no influence of the duration of storage on the success rate of cryopreserved oocytes/embryos.

9. Child welfare assessment should be done at the time of using oocytes/embryos for conception [43].

6.9 ESHRE guideline (2020)

The last version of the guidelines has been published by the European Society of Human Reproduction and Embryology (ESHRE, 2020) [60]. In this guideline fertility preservation has been discussed in women and transgender men concluded assessment before FP, the interventions, and also the post-treatment recommendations. It includes 50 evidence-based recommendations which were approved by the ESHRE Executive Committee and the Guideline Group. They consider this version needs to be updated 4 years after this publication.

This guideline considers intrinsic and extrinsic factors for the patients' assessment and selection. Intrinsic factors such as patient's health status, age, consent obtaining, and ovarian reserve assessment, and extrinsic such as the risk for fertility, and risk for pregnancy, … are discussed. The needs and ways for "ovarian reserve testing" are argued. Some of the important recommendations regarding OTC have been brought below.

In preparing a guideline, it's the first time that OTC methods have been reviewed, and the slow-freezing protocol is considered as standard protocol and offered the vitrification protocol only in the research program. One of the limitations of the present guidelines is the lack of discussion about the best cryopreservation method, although the choice of the method is selected by the embryologist and based on the conditions and facilities of the specialized centers, the freezing method is needed to be discussed in the guidelines.

Summarized recommendations of the ESHRE (2020):

1. OTC is recommended for the patients who must receive the high-risk gonadotoxic treatment and the oocyte or embryo cryopreservation is not feasible, or when the patient selects it.

2. OTC may not be useful for patients with low ovarian reserve[5] or advanced age.

3. The efficiency of OTC in patients >36 years old is doubtful.

4. Patients who have a history of low gonadotoxic treatment or a previous history of chemotherapy, also can be considered for OTC.

5. There is no limitation for ovarian stimulation immediately after OTC

6. OTC can be done when oocyte pick-up is done, following ovarian stimulation only for research purposes.

[5] AMH < 0.5 ng/ml and AFC < 5.

7. Ovarian transposition also can be done when OTC is done in patients who need pelvic irradiation.

8. OTC cannot be considered the primary option in transgender men but can be performed as an experimental procedure during gender reassignment surgery.

9. Ovarian tissue cryopreservation and transplantation can be done in patients with genetic and chromosomal associated POI disorders, after genetic counseling and in a research approach.

7. Guidelines regarding the concerns of cancer reintroducing

One of the serious concerns regarding OTC and transplantation is the risk of re-implantation, by the possible existing malignant cells in the harvested ovarian tissue after remission and resulting in the exclusion of ovarian cancer or when there's a high risk for ovarian metastasis [45, 68, 69]. It is of paramount importance in the cases of hematologic malignancies, which are at the most risk of ovarian metastasis, with the transferring of malignant cells via the bloodstream, and the highest risk was found with leukemia [69].

Recently, there is a new idea that ovariectomy after chemotherapy may be resulted in tissue cancer cell-free and increase transplantation safety in hematologic patients [12, 59, 70]. To evaluate the presence of leukemic cells in cryopreserved ovarian tissue histology and immunohistochemistry can be used to identify the ovarian infection by the malignant cell, but the molecular analysis is a better sensitive way. In leukemia patients, polymerase chain reaction (PCR) can reveal malignant cells in up to 75% of cases of thawed ovarian tissue [12]. RT-qPCR, and long-term (6 months) xenografting to immunodeficient mice, also have been used, in this aim [69, 71]. It must be mentioned that a disease-specific molecular marker may not be found in some cases of ALL, and AML [12]. In one study immunohistochemistry showed no evidence of the presence of residual leukemic cells, in the ovarian tissue after freeze and thaw. But the malignant cells were identified in 6 of 8 specimens, by the RT-PCR [72]. In the other study, it was shown that in the ovarian tissue harvested from ALL patients, there was no evidence of malignant cells in the ovarian grafts into immunodeficient mice, or in any of the other tissues, it's despite 4 RT-PCR positive malignant cells of the 7 grafts [73].

However, in a report in 2013, the author strongly discouraged ovarian autotransplantation when there's a risk of malignant cell reimplantation, especially in leukemia cases, and suggested patients consultation before reimplantation, in other cases in which the estimated risk is low [69], in the later publication in 2016, it was suggested that leukemia survivors also may benefit from OTC after providing maximal safety measures. These measures include harvesting after chemotherapy and an exact and intense search for leukemia cells within the graft. These cases must be informed that to date tissue involvement with malignant cells cannot be ruled out entirely with no measures [59]. Studies are needed to reassure the safety of this technique in different types of cancer.

In leukemia survivors, for ovarian tissue transplantation, careful consideration and evaluation must be taken to increase safety. Informed the patients thoroughly regarding the limitations of present investigation modalities, and the risk of cancer re-introducing should be weighed against the opportunity of being a biological parent [12].

8. Is ovarian tissue cryopreservation and transplantation still experimental?

Until recently, and for many years, ovarian tissue cryopreservation has been considered an experimental method, in many guidelines [15, 49, 50, 74]. One study described that the studies on tissue cryopreservation are limited, and most of them were done with no control group. There's a need for more experience, to accept this technique as a routine technique for fertility preservation. They said the published success of pregnancies may be because of the patient's native ovarian tissue [75]. In one study, in 2018, authors discussed this subject with more caution, and said because a live birth has not been achieved in the woman who had ovarian cryopreservation in the prepubertal stage, the efficacy is not still approved in children, and also in women with leukemia, the safety of this technique is still poor, so they considered it as experimental in children and in adults' patients which the risk for ovarian contamination with malignant cells is high.

For a technique to be defined as experimental or established some clear criteria are needed. European Society of Human Reproduction and Embryology proposed a platform to classify a technique as experimental, innovative, or established. The suggested applying criteria were efficacy, safety, procedure, and effectiveness, which all of them must be fulfilled, to accept a new treatment as established. These criteria also can be applied for the classification of the OTC and OTT in children, adult malignant cases with a high risk of ovarian contamination with malignant cells (e.g., leukemia), or low risk of ovarian involvement (e.g., breast cancer or Hodgkin lymphoma). In final, they classified this technique as established and no longer experimental when the risk of ovarian metastasis is low [48].

The first time, in 2016, in a study which was published by ASRM, it was concluded that there are enough national ethical and professional authorities for considering OTC as a standard modality, in fertility preservation programs [59]. Also, CSGE-ISGE (2018) mentioned that now there is large evidence of data that can confirm the effectiveness of the OTC, and it must be considered as a clinical and standardized procedure that would be promoted as soon as possible. The cryopreservation indications are extensive, and it is the only chance for prepuberty girls and patients who need emergency treatment (radiotherapy or chemotherapy) [45].

After many evidence-based studies and live birth reports, following transplantation, it's time to consider this technique as a clinical approach for medical indications (by the American Society of Reproductive Medicine), which can restore both patients` fertility and ovarian endocrine function [76]. Subsequently to more and more achievements of OTC reports, in the literature, researchers concluded that we passed the experimental phase.

In one study, the pregnancy rate per re-implantation was calculated, in the presence of evidence. In this large series of 111 cases, which the results were collected from Denmark, Belgium, Spain, Germany, and also from Australia, 29% of the women conceived (n ¼ 32). Knowing the exact number of transplantations, the data was highly relevant and evidence-based. This study approved the efficacy of the OTC technique [9].

According to one of these studies on 20 cases, the success rate for restoration of hormone activity was calculated at 94% [77]. Other studies, also referred to the worldwide live birth rate of 30–70% and concluded that this is a rigorous document to consider OTC as a standard protocol, for female fertility preservation options [36, 77, 78]. Many pioneers believe that now enough evidence support OTC and we reach the time to stop considering it as an investigational or experimental procedure [79].

9. Conclusions and recommendations

While the aim is to gather more evidence of the efficacy of the OTC, it is essential to consult the children and their families about this chance. There is no doubt that OTCP will be accepted as a successful standard method, soon with the fast growth in the technologies. They may suffer by delaying the offer.

We must discuss with all the patients, who may be at risk for premature ovarian insufficiency, following chemo- or radiotherapy, about the FP techniques. Discuss FP using OTCP, to the patients who do have no enough time for ovarian stimulation, just the time the diagnosis is established.

Patients should be well-informed about the different choices to be able to make valuable decisions according to the available evidence, as it may be their only real chance to be genetic parents in their future lives. A multidisciplinary team is needed, that could support all the necessary aspects of the FP, such as clinical, psychological, as well as legal, and ethical issues. After a comprehensive review of the present literature, a flow chart of the guidelines regards to OTC for cancer patients has been provided (**Figure 1**).

Regarding future FP programs, it must be stressed that however, the emerging options are promising, but their efficacy and safety of both established and newer techniques are needed to be confirmed by rigorous clinical trials, maybe with a focus on live birth, before introducing them as international clinical standards, and then can be offered as a medical intervention. Moreover, the exact criteria and relevant indication must be identified, in the aim to achieve the most efficacy of each FP method, also to reach more advancement in this field more research is needed on both established and newer techniques. Recent evidence has approved ovarian tissue cryopreservation as one of the promising options for female FP.

In the aim to give the patients appropriate and good clinical practice medical intervention about FP, widening access to the FP options and technologies is the key aspect (both appropriately patient selection and relevant indication).

However, negligence in referring the true cases to fertility preservation will result in depriving someone to have a family, applying FP to all cases, even with a low risk for gonadotoxicity, creating a large amount of unused stored reproductive cells and tissue, which burden costs for both patients and health services.

However, there are some new treatments in oncology with unknown gonadotoxicity effects, the risk of ovarian gonadotoxicity can be estimated in specific treatments which enough studies were done, and with sufficient detail, also ovarian reserve testing may be helpful, however, they have limited authority for predicting future fertility. So decision-making for many patients requires multidisciplinary discussion to evaluate the risk and benefits of FP interventions exactly. Providing comprehensive information is crucial to support the patients to have true decision-making.

Despite some general limitations in the current review of the published guidelines and the limited provided evidence, there is a hope that this document will help best practices in female FP.

From all guidelines, the following recommendations can be extracted:

1. It is needed to discuss with the patients the possibility of impaired fertility following cancer treatment.

2. The early referral to a reproductive specialist is a promising approach that gives a chance to improve fertility outcomes while minimizing the cancer treatment delay

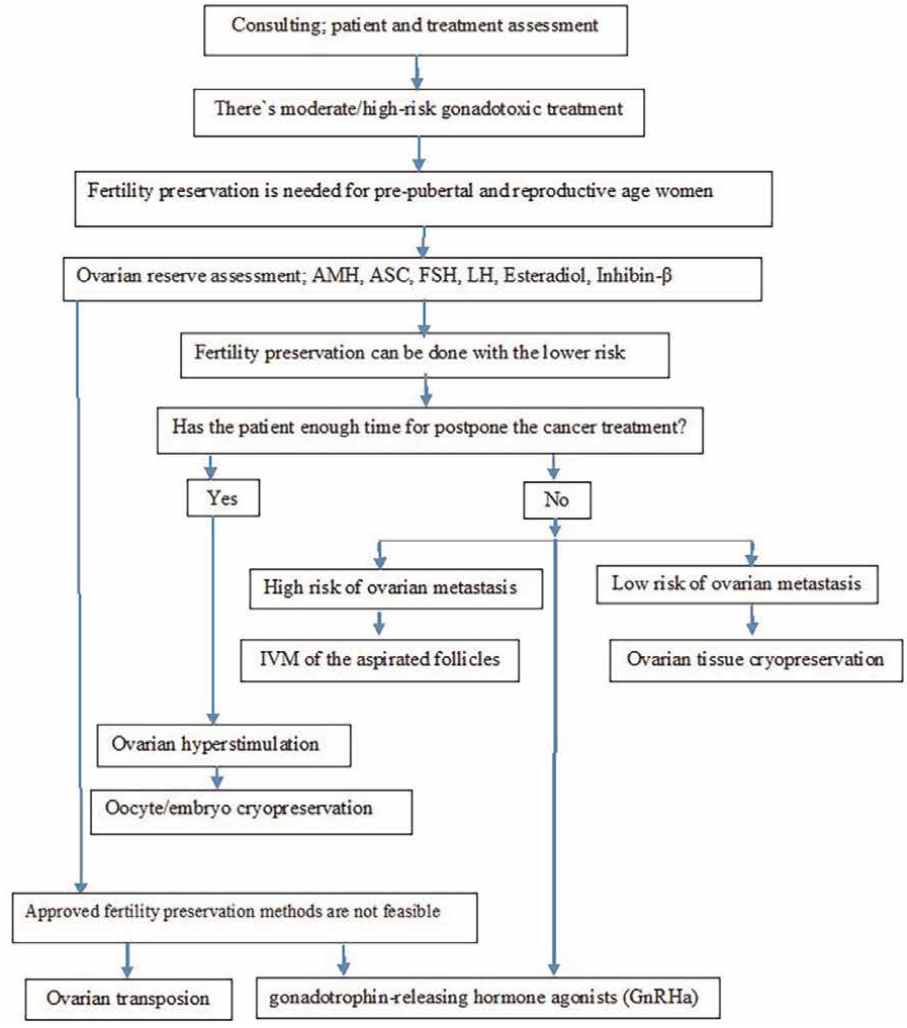

Figure 1.
Flow chart of a comprehensive guideline for referring cancer patients for fertility preservation.

3. It is necessary to identify malignant and benign conditions which approve the selected patient as a true candidate for ovarian cryopreservation.

4. Keep in mind all the current available established and experimental options to preserve female fertility

5. The difficulty in accurate estimation of the future impaired fertility in all cases

6. The difficulty in providing an accurate estimation of the risk for re-implantation after remission in blood-born hematologic cases.

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Chapter 5

Scaling up Cryopreservation from Cell Suspensions to Tissues: Challenges and Successes

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Abstract

This chapter covers the key physical, biological and practical challenges encountered when developing cryopreservation protocols for larger biological structures and examines areas where cryopreservation has been successful in scaling to larger structures. Results from techniques being used in attempts to overcome these challenges are reviewed together with the indicators for future development that arise from them. The scale-up of cryopreservation to tissues with diverse functions and cell types makes the control of freezing and thawing more challenging. Technology may—or may not—be available depending on the size of the material involved. To meet the challenge there must be innovation in technology, techniques and understanding of damage-limiting strategies. Diversity of cell structure, size, shape and expected function means a similarly diverse response to any imposed cryopreservation conditions and interaction with ice crystals. The increasing diffusion distances involved, and diversity of permeability properties, will affect solutes, solvents, heat and cryoprotectant (CPA) transfer and so add to the diversity of response. Constructing a single protocol for cryopreservation of a larger sample (organoids to whole organs) becomes a formidable challenge.

Keywords: cryopreservation, tissues, organs, slow cooling, diffusion, cryoprotectants, ice

1. Introduction

Historically, the predominant application of cryopreservation was in agriculture and reproductive medicine, starting with stored spermatozoa in the 1950s and oocytes being widely cryopreserved beginning in the late 1980s [1–3]. In the past decade, a revolution in tissue engineering has changed the landscape of cryopreservation and there is now a growing and critical need for successful cryopreservation of somatic cells not only as low volumes of cell suspensions but also in larger quantities and, increasingly, as part of a complex cell network. In such a network, different cells may have a range of different functions and structural requirements [3–6]. These larger subjects can contribute directly to a therapeutic treatment or can be

cryopreserved as tissue from which cells can be isolated to begin a manufacturing process [5, 7, 8]. A new demand has, therefore, been created for cryopreservation of larger subjects ranging from cell spheroids and organoids to tissue slices and, eventually, entire organs [3, 4, 6–11]. The potential benefits of cryopreservation of these multicellular and differentiated structures range from facilitating population-wide biopsy studies to supporting large-scale manufacturing and providing economies of scale within organoid preservation. Realising these benefits would support a sizable fraction of the needs of regenerative medicine and would advance progress towards organ cryopreservation, a key and as yet unmet need in transplantation technology.

The first steps towards large-volume cryopreservation must necessarily exploit the knowledge gained from the widespread, successful cryopreservation of cell suspensions [12, 13]. This success stems from the level of control of pre-treatment, cooling, warming and recovery that can be exerted over the cells [14, 15]. Appropriate control is supported by a specific technology, including programmable freezers and mathematical modelling, and benefits significantly from the relative uniformity of cell size, shape and cytoplasmic content of the majority of cell types of interest [15–19]. The important diffusion distances for solutes, solvents and heat are short for these suspended cells with little cell-to-cell differences and so provide relatively uniform responses to imposed conditions. Responses to applied cryoprotectant (CPA), whether physiological, osmotic or related to toxicity are also relatively uniform within a single cell type [20]. Additional complications that are introduced by a relatively large bulk volume of suspension, such as heat transfer across the sample, can be modified by altering the geometry of the sample, e.g., by flattening a cryobag containing suspended cells during cooling and warming [6, 8, 16].

While there is a promise with ice-free techniques, also known as vitrification, these have been covered in other reviews and so will not be examined here [21, 22].

2. The cryobiology of scale up

2.1 Practical challenges

Significant challenges that arise when moving up to the cryopreservation of large, coherent cell masses are caused directly by the size and volume of the tissues concerned. As noted above, in a cell suspension the diffusion distances between the cytoplasm and surrounding medium are effectively constant for each cell, ensuring relatively uniform responses to imposed physical and chemical diffusion gradients, such as external cooling and CPA addition. For larger cell masses such as organoids, a much greater range of diffusion distances exists because cells further towards the centre of the structure are increasingly distant from the external medium [6, 8, 17, 18, 23, 24]. Rates of diffusion for these cells are further complicated as diffusion within the overall cell mass will involve transfer across adjacent cells and intracellular spaces, with a range of differing properties before the external medium is reached [4, 24].

For numerous mammalian cell types (including cells derived from blood, liver and ovaries), cryopreservation of suspensions is straightforward with limited loss of cell viability and function [12]. Moving up in size to single cell-type spheroids this success is often continued, as in the case for liver spheroids [8, 25, 26]. As biological structures become more complicated, e.g., cell organoids composed of several different cell types, success is more limited with a strong, negative influence of size [4, 6]. Many smaller, immature organoids, typically consisting of no more than a few hundred

Biological Sample Type	Cryo-preparation and state	CPA	Cooling rate/ method	References
HepG2 liver spheroids	HepG2 spheroids in alginate, spheroids of a few 100 s cells	12.5% Me_2SO	0.3°C/min	[8]
Testicular tissue	Cut into 2–5 mm segments	8% Me_2SO; 20% Serum	1°C/min (Mr. Frosty Passive cooler)	[7]
Intestinal organoids	Disassociating into individual crypt colonies improved outcome	10% Me_2SO; 10% Serum. Y-27632 ROCK inhibitor improved outcome without disassociation	Not stated	[4]
Prostate organoids	Multi-organoid structures broken up by pipetting	10% Me_2SO	1 °C/min (CoolCell Passive cooler)	[28]
GI organoids	Biopsies cut into 2–3 mm^3 cubes	10% Me_2SO	1 °C/min (Mr. Frosty Passive cooler)	[29]
Ovarian biopsies	Divided into 2–3 mm^3 fragments	10% Me_2SO	1 °C/min (Mr. Frosty Passive cooler)	[30]
Neural organoids	84 days old, <1mm^3	Methylcellulose and Me_2SO (concentration not stated)	1 °C/min (Mr. Frosty Passive cooler)	[27]
Whole sheep ovaries	10–15 cm inc. vascular pedicle	1.5 mol l^{-1} Me_2SO, 0.1 mol l^{-}1 sucrose and 10% serum, organ perfused	0.2 °C/min (−9 to −40°C)	[31]
Thymus	1 mm thick strips	10% Me_2SO	1 °C/min	[32]
Whole sheep uteruses	40 g	10% Me_2SO, organ perfused	0.2 °C/min	[33]

Table 1.
A summary of some tissue types currently cryopreserved in the presence of ice successfully, and the methods used to achieve results.

cells can be cryopreserved [27] but for larger, mature organoids in their final state for therapeutic use, cryopreservation success is more limited. **Table 1** summarises some successful strategies for a range of such tissue types.

For even larger tissues and whole organs, success is largely limited to those which can operate as discrete units when dissected, for example, ovarian tissue and thymic slices [31, 32, 34, 35]. These can be removed from the body and cut into smaller functional units, which can each be successfully cryopreserved, thawed and transplanted independently. Mammalian organs lacking this ability such as the heart and kidneys cannot, as yet, be cryopreserved successfully [9, 10]. A famous 1978 paper on the subject started with the line '*Attempts to preserve viable kidneys by freezing in the presence of cryoprotective agents have been notoriously frustrating*'—a statement no less true today than it was 45 years ago! [36]. The ability to cryopreserve elements of structure and function in excised tissues also has clear medical benefits when applied to biopsy samples. For microscopic investigation where the function is not required then

structure/tissue architecture is of primary concern [37]. Conversely, when functional assessment is required, then balanced and optimal cellular performance must take precedence over structure. This indicates an interesting, and valuable, halfway house for cryopreservation where success can be measured in terms of either the structural integrity or function of recovered material [37, 38].

Cell therapies and regenerative medicine treatments require methods that offer successful cryopreservation, are practical and meet regulatory requirements if they are to form parts of medical devices and/or require cGMP manufacture [14, 26, 39, 40]. This can become an issue for larger samples if novel, larger sample containers need to be devised to facilitate effective processing, including CPA treatment, cooling and warming. For example, only cryobags and hermetically sealed cryovials are permissible for cGMP therapies. The latter enables simplified aseptic filling operations and typically has thickened plastic walls to prevent damage at low temperatures. These thick walls limit the heat transfer rates achievable and so may influence the design of the cryopreservation protocol [41]. On the contrary, cryostraws, commonly used in reproductive medicine, have internal diameters in the order of 1–2mm that increase their surface-to-volume ratio for more efficient thermal transfers of the sample and so they are only suitable for the smallest spheroids and organoids [42]. Regulations of course vary between regions, but broadly align when the manufacture and use can take place over multiple jurisdictions, requiring compliance with all regulatory regimes [14, 40, 43].

It is important to accommodate such practical difficulties into the initial design of the cryopreservation protocol as retro-adapting methods for clinical delivery once they have been developed are lengthy and costly and can delay (and in some cases prevent) a treatment gaining widespread use. Other issues such as a need for automation during processing may also have an impact [14, 40].

2.2 CPA loading and unloading

An early event where the extended diffusion pathways of larger structures are evident is in the loading and unloading of permeating CPAs such as Me_2SO [6, 11, 17, 18, 24, 34]. Following the addition of a permeating CPA an initial, cellular response of exposed cells is to shrink due to the osmotic gradient the CPA exerts [34]. As the CPA then permeates into the cell, the gradient is diminished and cell volume recovers to a significant extent [34]. In larger structures, exposure to the gradient, and the responses to it, will be delayed for those cells embedded deeper in the structure [44]. This generates a risk of insufficient CPA protection if cooling proceeds before CPA equilibration is reached in the central regions of the structure. However, an extended incubation time in the CPA to ensure deep equilibration can lead to damaging levels of toxicity for more peripheral cells. The larger and more complex the structure the more challenging this issue becomes, with both extracellular channels, cell membrane parameters, viscosity, temperature and physical distance all playing a role [19, 24, 44]. A similar issue, but reversed in direction, is encountered on warming and subsequent CPA removal [18].

Tissue architecture can provide additional complications for CPA treatments. For example, mature organoids may contain a central cavity devoid of cells, or with a different cellular composition [27, 45] and sufficient time for CPA diffusion into this cavity is necessary to prevent further CPA diffusion from the innermost cells into the cavity following cellular equilibration. This would result in an overall CPA loss from the inner cells, compromising the chances of achieving the required level

of their post-thaw cell survival to maintain organoid integrity. Chondrocyte and cartilage samples, typically cryopreserved with bone attached, provide a further example. As commonly used CPA cannot pass through bone, this further limits the surface area for diffusion of water and CPA, restricting diffusion pathways and transfer speed [17, 23].

Several methods have been employed to alleviate CPA loading and unloading difficulties that may prove to be applicable if modified for larger structures. One such method involves adding an initial CPA concentration to the external medium that is higher than that considered necessary for successful cryopreservation. As CPA diffusion is driven by concentration gradients, this higher concentration external to the biological sample will increase the CPA diffusion rate and thereby reduce the required incubation time. When the tissue is calculated to be sufficiently protected, the extracellular CPA concentration can be reduced to its equilibrium value [17, 23, 44]. Such methods are more often used with systems preserved through vitrification (ice-free cryopreservation) but are equally useful to overcome CPA loading issues in slow-cooling techniques. However, the high concentration of CPAs, at the relatively high incubation temperatures employed, can cause significant cytotoxic responses in sensitive cells near the outer surfaces of a larger structure. The temperature could be reduced to lower CPA toxicity, but as viscosity is temperature dependent [46, 47], any lowering of temperature would increase incubation times to achieve the required level of diffusion, thereby negating any benefit of the lowered temperatures. Using a mixture of different CPAs can reduce the concentration, and so toxicity, of any one given CPA can also be used to mitigate this problem. Such techniques are common in large-volume vitrification and may help in slow-cooled systems with long incubation times [20, 48, 49].

When working with entire organs in which the circulatory system is intact, the blood vessels can be perfused to reduce CPA distribution time and ensure homogenous CPA loading [35]. Perfusion is an established technique in major surgery and organ analysis [50] and the replacement of blood or stabilising solutions with CPAs can effectively reach areas of tissues difficult to reach by diffusion or surface-induced effects alone [21, 31, 35, 51]. This has shown to be effective in some cases [21, 31, 33, 51], yet most studies focus on the very high CPA concentrations required for vitrification that are currently less applicable to larger structures using slower cooling rates. The systems involved may be susceptible to vasculature cryoinjury, with damage to small blood vessels during cooling, sufficient to prevent effective CPA removal resulting in necrotic areas after thawing due to CPA toxicity. These methods are also limited to tissues with the full circulatory system—immune privileged tissues without vasculature cannot benefit from this technique—and require specific technical skills to perfuse the organs successfully.

Extracellular CPAs, which can help dehydrate cells and protect cell membranes pose particular problems for larger structures as they will only protect the outermost cells of the structure, or ones that can be reached through extracellular liquid channels. Innovative methods to exploit the potential benefits of perfusion to slow cooling techniques are required.

2.3 Diffusion of heat and intracellular water

In a suspension of separated cells undergoing cryopreservation, the diffusion distance for heat, water and solutes between individual cells and the external medium is no larger than the radius of a cell. Additionally, diffusion of water and intracellular

CPAs is influenced by membrane permeability to these compounds and the cell surface area to volume ratio. These factors will vary in differing, but limited ways when small-cell aggregates are present. Having relatively uniform characteristics means that the cellular responses of single cells, and small-cell aggregates, to imposed thermal or chemical gradients will be similarly uniform, providing the level of control needed for successful cryopreservation. As noted above, the consequence of working with larger, multicellular structures is that the diffusion pathways are extended and depend on the dimensions of the cell mass. They will also involve transfer across a number of cells and extracellular space [11, 24, 44]. The location of individual cells within the cell mass and their type—each with their specific membrane permeability coefficients and surface area to volume ratios—will influence their response to any imposed diffusion gradient over time and so the level of overall control of heat and water and diffusion of CPAs will be diminished.

Some dehydration may occur in response to CPA treatment in the initial phase of the preservation protocol but the greater part occurs once the ice has formed in the system [41, 44]. This is referred to as cryodehydration. During controlled, slow cooling the extracellular solution commonly falls below its melting point, entering a supercooled state, before ice forms by spontaneous, or induced nucleation [52–54]. When ice nucleates there will be a temperature discontinuity (an exotherm) within the system related to the release of latent heat of freezing accompanied by a sharp increase in the osmolality of the extracellular medium as water molecules, and only water molecules, become components of ice crystals [55]. Biological material is excluded from the crystal lattice [52]. The nucleation event initiates protective cryodehydration, as described above, but if supercooling is extreme prior to nucleation then the large and immediate osmotic shock delivered once ice forms can be damaging to the sample. The overall size of a sample (tissue mass plus cryomedium) influences ice nucleation and the larger the volume the earlier ice nucleates [52, 56].

Once nucleation has taken place, cells in suspension become entrapped in channels between ice crystals and cellular dehydration is primarily limited by their membrane permeability to water [54, 57, 58]. This protective cryodehydration during cooling is essential as cells retaining a high intracellular water content are more likely to experience lethal intracellular ice formation (IIF) than their more dehydrated counterparts [11, 59, 60]. Cells that have a high membrane permeability to water can survive relatively rapid cooling as water is able to leave the cell quickly enough to prevent IIF. However, at lower temperatures cell permeability decreases, the level of this reduction being cell type dependent. The lower the permeability the slower cooling must proceed to ensure sufficient dehydration occurs, with 1°C/min after ice nucleation being a typical value for somatic mammalian cells in suspension [41, 54].

Larger structures will become embedded in the matrix of ice crystals after nucleation. In a cell spheroid, for example, not all the cells are at the outer surface and so, rather than dehydrating directly into the cryoprotective medium, some cells will transfer water to those in physical contact with them that generate an osmotic gradient, and only those at the outer surface of the sphere will interact directly with the extracellular medium. The overall dehydration rate for the spheroid is, therefore, slower than would be observed for single cells in suspension and, inevitably, the fastest acceptable cooling rate for cryopreservation of the biological sample will also be lower. However, as the slowest cooling rate is essentially defined by the sensitivity of the cell type to CPA toxicity, this remains unaltered, resulting in a narrowing of the range of acceptable cooling rates for successful post-thaw survival [59]. In some

instances, a lower recovery rate than is seen in suspensions can be the consequence of the slower rate for the complex system—the highest survival after optimisation being lower than the value achieved in suspensions. In HepG2 liver cell spheroids, the optimal cooling rate falls to 0.3°C/min from 1 to 2C°C/min for a cell cluster of a few hundred cells [8]. This problem becomes more pronounced in organoids containing multiple cell types where dehydration will be limited by the cells with the lowest membrane permeability—the maximum cooling rate becoming increasingly slower as the biological structure becomes larger and more complex. The solution to this dehydration problem is likely to be to lower the cooling rate, where this does not impact post-thaw cell functions. Most somatic mammalian cells can tolerate a relatively low cooling rate, down to 0.1–0.3°C/min, which is usually sufficient for dehydration to occur. T cells for example have shown similar optimal survival at rates of 1°C/min and as low as 0.1°C/min [41], and ovarian tissue samples are typically cooled at rates of 0.2–0.3°C/min [31, 61–64]. As can be seen in **Table 1**, most spheroid and organoid cryopreservation methods currently use passive coolers, where control of the cooling rate is limited and producing rates in the vicinity of/of approximately 1°C/min—moving to controlled rate freezers with lower and more precise rates would allow for more precise control over cell dehydration [15].

Ice formation can be physically damaging for cell suspensions when the cells become trapped in channels between crystals [58]. At higher temperatures, the channels are relatively wide, and the cells have minimal direct contact with ice crystals, minimising the potentially damaging effects of distortion, crushing and shear forces. As the temperature falls, more water molecules are locked away as ice and the channels reduce in size [54, 55, 58]. Larger samples are at an increased risk of direct contact with ice under these circumstances, resulting in damage that can impact negatively on recovery. Relatively delicate tissues such as spheroids and organoids can be crushed in this way. Extracellular ice also damages complex tissue structures by disrupting cell-cell contacts, and thereby damaging intercellular communications. Severing these connections is not only damaging to individual cells, it can also reduce the overall function and communication between the surviving cells tissue or organoid.

Different CPAs can be used, perhaps in combination, to help with dehydration difficulties with larger samples. Where lower cooling rates are not practically possible or biologically tolerable with only the permeating CPA Me_2SO, then dehydration can be accelerated through the use of extracellular CPAs such as sugars [3, 20, 31, 64]. These CPAs decrease the osmotic potential in the extracellular space, and so can drive more rapid dehydration. This may offset the effect of a lower surface-to-volume ratio of spheroids and organoids relative to individual cells. The addition of different types of CPAs, such as apoptosis inhibitors to the cryopreservation and post-culture medium, has been shown to improve organoid survival in some systems [4]. Altering the size and shape of samples where original structure and integrity are not the priority can also improve the outcome. Ovarian tissue for example is often cryopreserved in strips to maximise the dehydration rates as these tend to be more effective than spheres due to the larger surface area they provide, and an increased surface area can improve biological outcomes [63, 64]. However, in many cell types, when it comes to large, mature organoids containing several cell types the problems faced by dehydration issues cannot easily be overcome. Intracellular ice can still form and be lethal and more research is required to increase dehydration rates, or perhaps lower the possibility of IIF even at relatively high cell hydration levels.

2.4 Ice nucleation and direct ice damage

A further issue with extracellular ice formation is the increased volume of ice crystals—when ice forms it expands to occupy approx. 12% more volume than the liquid state. In cell systems such as organoids, the formation of ice in the internal, liquid-filled cavity, can generate sufficient mechanical pressure on the cells lining the cavity to cause significant fractures. This can disrupt the organoid structure, yet individual cells may survive the cryopreservation procedure.

Intracellular ice is lethal for the cell in which it forms but in a cell suspension, where the cells have limited direct contact with each other, a frozen cell rarely nucleates others. A proportion of weakened or damaged cells in the suspension will experience intracellular freezing but this poses little risk for the greater cell population. However, in a larger structure where cells can be tightly pressed together and/or physically interconnected, ice that forms in one cell can spread to another [65]. This triggers a chain of intracellular freezing throughout the structure that can cause significant damage and cell mortality. Strong evidence of the damage that can be caused by ice comes from tissues and organs which survive cryopreservation, at least in part, with slow cooling. Excised ovarian and thymus tissues are notable in this regard and are dealt with in more detail below. The impact of this damage has been observed in thymus slices, cryopreserved at 1°C/min in 10% Me_2SO [32].

A good example of this chain reaction of cell-to-cell ice growth is seen when considering the studies presented by Ross et al. [32]. In this work, histology was carried out (H&E staining) to detect viable tissue and areas of autolysis (indicating cell death); autolysis was seen over continuous areas with some completely devoid of surviving cells and other areas with almost total survival. Autolysed areas form in different places in different samples and so are not related to location in the tissue or placement in the vial in which it was preserved. In a thawed tissue, mass with limited intercellular connections, living and dead cells would be expected to be distributed relatively uniformly throughout the tissue. The aggregated areas of autolysed cells observed suggest there was a significant intercellular connection (as far as required for ice to spread) within the tissue and that once intracellular ice nucleation occurred in a small number of cells it spread rapidly to conjoined neighbours. When thawed, these slices were transplanted into an athymic mouse model where they were able to support T-cell development, showing preservation of function [32].

In certain circumstances, supercooling techniques have been proposed as an alternative cryopreservation method that avoids ice and its associated lethal impacts. Supercooling involves cooling a sample to high sub-zero temperatures, typically between 0 and − 10°C, under conditions where ice is relatively unlikely to form thermodynamically. At such temperatures, biological activity is reduced and both structure and function can be protected for several days. Whilst such a short timeframe is limiting, this can be sufficient to overcome extreme time constraints associated with, for example, transport, quality checks and organ transplants [11, 62, 66–68].

2.5 Control of ice structure: a way forward?

Ice damage is generally accepted to be the most severe and the leading cause of cryopreservation-related injury and cell death in large biological tissues [11, 21, 60, 69] and can be considered the most difficult problem to overcome. However, ice crystal structure is not constant [41, 58] and new ways of manipulating ice growth may help reduce the damage it causes.

One of the simplest ways to change ice structure is by manipulating the cooling rate, especially in the high sub-zero zone where most ice forms (c. −5 to −40°C) [41, 47]. In **Figure 1**, the ice structure of a 10% Me_2SO solution is shown for samples experiencing cooling at 10°C/min; cooled at 1°C/min and at 0.1°C/min. These rates were those recorded after ice was nucleated at −4°C. At very low rates of cooling where ice growth rates are also very slow, the ice has time to organise into large crystals—the most thermodynamically favourable state. Research is limited as to how different forms of macroscopic ice structure impact cryopreservation; however, slower rates of ice growth are known to inhibit damaging ice-recrystallisation on thawing and reduce the osmotic pressure on the cells as the rate at which water molecules are locked into any recrystallising ice is reduced [41, 70]. The ice structure is very different at 1°C/min, a typical cooling rate for cell suspensions, compared with cooling at the much slower 0.1°C/min.

There are some indications that by using very low rates of cooling, more structure can be preserved. **Figure 2** shows the whole mouse embryonic kidney, heart and liver cryopreserved at only 0.2°C/min in 12.5% Me_2SO. As can be seen in the figure, these organs (2–5 mm max. dimension) had good post-thaw structure.

Figure 1.
The structure of ice in a 10% Me_2SO saline solution in a cryomicroscope at 10x magnification after controlled cooling at different rates to −100°C. samples were cooled, left to right, at 10°C/min, 1°C/min and 0.1°C/min. The extremely low cooling rate used in C results in a markedly different ice structure.

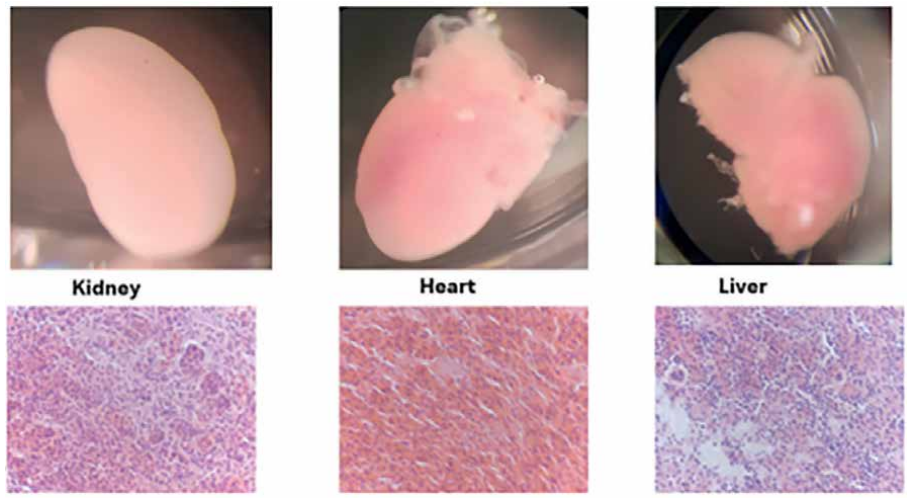

Figure 2.
Mouse embryonic kidney, heart, and liver after cooling at 0.2°C/min and storage in LN for >30 days. The overall structure of the organ (top), and histology (bottom, H&E stain) of the tissue indicate minimal cell and structural damage on cooling.

New developments in cryopreservation technology allow ultra-slow cooling rates and long cooling times, and so open the door to new ice structures—mammalian nucleated somatic cells tend to be robust to very slow rates of cooling. Many of the large tissues currently cryopreserved use very slow rates of cooling—ovaries at 0.2 or 0.3°C/min [31, 63, 64], liver spheroids at 0.3°C/min [8] and uterus at 0.2°C/min [33] —while this will help in dehydration and CPA diffusion as discussed above, the different ice structure in these ultra-slow cooling regimes likely plays a role.

Historically, the manipulation of cooling rates was seen as a key parameter to the successful cryopreservation of whole organs. A 1984 study found that extremely low rates of cooling, as low as 1°C/hr. in this case, resulted in better vascular resistance readings, tissue architecture observations, with ice seeming to have been localised to extracellular zones more at these slower rates of cooling [71]. Microscopic studies using freeze-substitution paint a similar picture [72]. Such slow rates of cooling have been scarer in recent years, partly due to the practical difficulties of applying low cooling rates at the time, and due to fewer needs for larger structure cryopreservation. Applying these exciting but somewhat neglected methods to modern tissue-engineered structures and organs, along with combining them with new cryoprotectant knowledge and technologies offers perhaps the best chance for widespread tissue preservation.

Ice structure can also be effectively manipulated through the introduction of ice nucleation and ice-inhibiting particles, as well as cooling rates and CPA concentration [60]. Higher nucleation temperature tends to cause larger ice crystals as less of the freezable water solidifies at the initial point of nucleation, more supercooling—as is seen in the absence of ice nucleators—causes a smaller, more dendritic and ice structure. More viscous CPAs will slow the rate of ice crystals growth by inhibiting the diffusion of water molecules onto the crystal-liquid interface [19, 70]. In future, adapting parameters such as this may be able to reduce the damage caused by ice enough to allow for the preservation of a larger portion of a larger number of tissues.

3. Cryopreservation of larger structures: the special case of ovaries and thymus

The ovary consists of follicles at various states of maturity, in which immature oocytes reside. These follicles and slices of the mammalian ovary have been successfully cryopreserved [61, 63, 64, 73]. Ovarian tissue can be cryopreserved before cancer treatments which may damage the ovaries and can be thawed and transplanted when the patient wants to have a baby, allowing for natural conception [63, 64, 74, 75]. In human ovaries, the tissue is often cryopreserved in follicle-containing slices, which in addition to simplifying the physical problems of larger tissue cryopreservation, has the additional advantage that only a single slice has to be transplanted back at any one time, allowing for multiple pregnancies following separate thawing procedures. Ovarian tissue preservation can be particularly beneficial in pre-pubescent girls undergoing treatment where hormonal stimulation to produce mature oocytes for cryopreservation is usually not possible [63, 64, 74].

Carroll et al. [76] first published successful births in mouse ovarian follicles (a liquid membrane containing immature oocytes which are surrounded by layers of granulosa cells) in 1990. The method involved incubating the samples in Me_2SO (1.5 M) and serum for 10–12 minutes, then seeding ice at −7°C and followed by a cooling rate of 0.3°C/min [76]. By 2014, Campbell et al. cryopreserved whole sheep

ovaries, which were able to produce fertile offspring after thaw and re-transplantation [31]. For these larger tissues, the ovary was first perfused [35] using the blood vessel architecture with CPAs (Me_2SO, calf serum, and extracellular CPA sucrose, for up to 60 minutes, and cooling proceeded at only 0.2°C/min). The success of these techniques shows that, with sufficient CPA incubation, appropriate cooling rates and controlled ice nucleation, then larger structures can be preserved with widespread success [61–64, 75, 77]. It is observed that tissue that can be physically sliced and still function on transplant can also survive cryopreservation, and tissues that cannot be sliced and survive do not survive cryopreservation. This may indicate that physical damage due to ice disruption within tissues is certainly a central issue in the cryopreservation of larger tissue samples.

Another tissue that can be cryopreserved with success is the paediatric thymus. Thymus transplantation is carried out to treat paediatric diseases such as complete DiGeorge syndrome, in which infants lack a thymus [78]. Thymus is obtained from a donor and sliced into up to 30 pieces, approximately 1 mm thick. These slices are then cultured to deplete the donor thymocytes (large numbers of donor thymocytes could potentially cause an immune reaction in the host), leaving mainly stromal and epithelial cells for transplantation. Transplantation is done in the well-vascularised thigh where circulating recipient progenitor cells are able to populate the transplanted slices and undergo T-cell development, eliminating the need for more complicated chest surgery where the thymus usually resides [32, 78, 79].

Cryopreserving such tissues will allow for the creation of thymic tissue banks, giving a supply of tissue on patient demand and allowing for future recipient tissue or partial tissue matching, surgery at the optimal time and location for the recipient. The authors have found that these samples can be cryopreserved at 1°C/min in 10% Me_2SO without the need for ice nucleation [32]. Rapid diffusion of water and solutes is facilitated by the slicing of the tissue pre-cryopreservation. The thymus does not have to be completely intact to fulfil its function of supporting T-cell development, so the areas of tissue that survive the freeze/thaw have sufficient capacity to restore the peripheral T-cell population in the mouse model [32].

4. Biopsies

An area of cryopreservation that is sometimes overlooked is that of biopsies. These small pieces of tissue, typically of the order of 1–3 mm^3, are cryopreserved for reasons ranging from diagnostics and cell extraction to fundamental research [7, 29, 30, 80, 81]. Typical cryopreservation of these structures involves direct plunging into liquid nitrogen without the use of CPAs [80]—this may allow the recovery of some markers and DNA but living cells and faithful tissue architecture is lost. A particularly promising use of optimising biopsy preservation is their use for population-wide studies where biopsies are taken from many patients over many years and stored in biobanks [11, 82]. For the most effective use of such biobanks, preservation methods should allow tissue architecture to be preserved, together with undamaged DNA and protein content, and for viable cells to be available for regrowth. This would open up the possibility of extracting an increased range of data from the samples as well as future-proofing samples for examination by techniques not developed at the time of preservation.

Using current methods, even with the use of CPAs and some control in cooling, liver biopsies can have recovery of oxygen consumption and mitochondrial

functions—something elusive with the whole organ [81]. Cryopreserving as tissue or at least as cell clusters may give better single-cell performance than tissue fully digested prior to cryopreservation [83].

Current preservation techniques can provide high level, tissue architectural preservation in organs as complex as the brain, and success has been reported in heart valves using ice-free methods (either vitrification or non-low temperature preservation) to preserve the structure [11, 21, 84, 85]. However, such methods tend to preserve only architecture and not viable cells. Accepting current technical limitations, the balance between preserving tissue architecture/structure or cellular function can be altered. Typically, the structure is the preferred option for biopsies with samples cryopreserved rapidly sometimes in the absence of CPA, resulting in near-total cell death. However, biopsies can also be used to extract living cells, typically for regenerative medicine and organoid culture [29, 30, 82], and slow cooling methods discussed above could allow for sufficient structural preservation as well as ensuring an acceptable recovery of some viable cells. A cryopreservation method where the structure is preserved but also allows for live cells to be extracted would enable considerably more data to be extracted from population-wide samples, markedly improving scientific efficiency and productivity. Overcoming these challenges with new techniques may require an initial focus on specific applications where known demand exists, for example, in biopsy preservation in cancer patients for extraction of tissue infiltrating lymphocytes. Success here might also provide valuable new knowledge relevant to the development of protocols for larger structure cryopreservation.

5. Conclusions and future direction

Cryopreservation is a rapidly developing field that is continually adapting to meet the challenges presented by ice and low temperatures when trying to preserve viability in larger tissues and structures. The larger structures become, the more challenging attempts at cryopreservation, using current techniques, becomes. It is possible that some methods, such as optimisation of known CPAs, may be approaching maximally optimised thanks to modelling (although the door to new CPAs and their reactions remains open), and most current knowledge gained from cell suspensions has already been applied. However, many relatively unexplored avenues of research are available and actively being explored to achieve a viable post-thaw outcome—combining these new techniques with the manipulation of ice structure from lower cooling rates shown to minimise ice damage [71, 72] is an obvious route forward.

There are also cryopreservation methods exploiting higher temperatures, such as supercooling discussed above. Taking samples below the appropriate glass transition temperature (as in conventional storage in liquid nitrogen) will provide dramatically extended storage time for samples, measured at least in decades. However, at a practical level, many applications may not need such a guarantee e.g. preparations for cell therapy or organ and tissue samples destined for application in the short term. Where storage of several weeks would suffice, for example, then storage at a relatively high temperature, where ice could be avoided or at least occupy a lesser fractional volume was harnessed, may provide significant benefit.

In some special cases, such as the thymus and ovaries, it is already becoming possible to cryopreserve mammalian organs and, in time, the number of these cases will doubtless grow through the development of new CPAs, new loading and unloading methods, and techniques to overcome the damaging effects of ice crystals.

While ice-free methods offer a promising, but more distant avenue for cryopreservation, slow-cooling methods enjoy current success and will likely form the key to the delivery of many cell therapies, tissue-engineered constructs and other larger tissues in the future.

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Section 2

Prospects of Cryopreservation as Applied in Blood and Plants

Chapter 6

Impact of Different Cooling Methods on the Stability of Peripheral Blood Mononuclear Cells (PBMCs)

Nahla Afifi, Eiman Al-Khayat, Linda Hannigan, Monika Markovic Bordoski and Israa Khalaf

Abstract

During cryopreservation of peripheral blood mononuclear cells (PBMCs), there are several recognized cooling methods, which include different cooling rates that might influence the stability of the PBMCs. This chapter will focus on three cooling methods trialled and will describe the different principles they are based on and the outcomes. One cooling method is based on repeatable –1°C/min cooling rate that requires only isopropyl alcohol (method A). The second cooling method is based on the cooling rate of –1° C/min solely (method B). The third cooling method is based on a user-predefined programmable controlled rate of freezing (method C). The first method was discontinued for safety reasons. A small comparative study was performed using 12 cell preparation tubes (CPT) using methods B and C. Cell Viability was measured based on the difference between pre-thaw and post-thaw viability percentages that were obtained from the flow cytometry. From our data, we conclude that although there were no significant differences in the outcomes of the comparative study of cooling methods, the use of either method B or C are the most suitable for long-term storage that will preserve the quality of the sample suitable for future research and clinical applications.

Keywords: cooling, PBMC's, stability, viability, pre-thaw, post-thaw

1. Introduction

PBMCs are white blood cells with round nuclei that includes lymphocytes (i.e. T cells, B cells, and NK cells), monocytes, and dendritic cells [1]. These cells are important biospecimens as researchers use them to recognize circulating disease biomarkers. While fresh viable cells are most often being used, the use of frozen viable PBMC's should be equally considered as they allow the screening of purified monocyte and lymphocyte populations. Denity gradient centrifugation has been utilized for isolating PBMC's because it is not expensive and needs very little specialised

Figure 1.
Whole blood is layered over the density gradient medium. The centrifuged blood is showing the cells in different layers based on different densities. PBMCs settle between the density gradient medium and the plasma, where they can be aspirated [4].

equipment to implement in any lab [1], following cryopreservation, in addition to, functional studies, immunophenotyping, obtain lymphoblastoid cell lines (LCL) by Epstein Barr virus (EBV) transformation, and purification of CD34+ cells [2].

Using cell separation techniques, PBMCs can be isolated directly from whole blood. They are present in peripheral blood, and they have a crucial function of acting as the body's front line to defend against disease [3]. PBMC isolation is based mostly on the method of density gradient medium, and centrifugation [4] as shown in **Figure 1**.

Density gradient centrifugation has been utilized for isolating PBMCs because it is not expensive and needs very little specialized equipment, to implement to any lab [1, 5]. The aim of this chapter is to investigate the effectiveness of different approaches used in freezing rates to ensure that PBMCs are maintained within the optimal viability and functionality, and to preserve the cells with higher viability and biological activity, before and after the thawing processes.

2. Literature review of different cooling rates on PBMCs stability

Our research in this field highlighted that there is a lack of literature about the effects of different cooling rates during sample storage. Some studies provided data that shows the crucial steps of sample storage and handling in maintaining the viability of PBMCs, the recovery of PBMC and T-cell functionality [6]. As such, a review of the available studies will be discussed.

2.1 Quality of frozen PBMCs

Researchers have identified that cryopreservation affects the viability, recovery, and gene expression pattern of PBMCs, when compared to freshly isolated PBMCs [7, 8]. In addition, multiple factors impact the quality of PBMCs including preanalytical, analytical and post-analytical processes. Pre-analytical steps such as the time of sample collection, environmental conditions and calibration of equipment [9]. Analytical steps included the sample processing, type and time of exposure of the cryoprotectant media, viable cells manual mixing conditions, sterile environment and freezing conditions are all critically important for good sample cryopreservation [6]. Post-analytical factors included transportation of viable cells is of paramount consideration as they affect biological specimen viability and functionality [2]. Also, temperature fluctuations happen during retrieval or shipping of stored samples [8]. Nevertheless, an essential step is that the collected PBMCs are conserved in a natural state that renders them from being altered functionally. PBMCs need to be grown in cultures to show viability and to react to immune stimulation to show

phenotypic capability [10] Best practices relating to the maintenance of PBMCs viability is obtained if they are stored below –132°C, the glass transition temperature of water (GTTW) [11]. At this temperature or below, the biological activities of cells are stopped [8]. Additional research suggested that quality control measures in cell repository should be adopted or based on their study findings. The separation of blood, and the storage using a controlled-rate freezer should be within six hours from collection. Environmental safety controls such as a temperature monitoring alarm system should be configured in the liquid nitrogen storage tanks. The study findings recommended to use liquid nitrogen vapor for maintaining a high cell viability through the storage for long-term purposes. However, using gasket threaded vials can also be used if storage is in the liquid nitrogen phase [12].

2.2 Different cooling rates vs. cell viability

PBMCs are being monitored through cell yield, viability, and cell population percentage using fluorescence flow cytometry. Research has concluded that it is important to disclose the temperature and time of processing when data from clinical trial of PBMCs is being published [13].

A study was done to show the impact of multiple temperature fluctuations on cell quality, PBMCs were stored under suboptimal storage condition from 10 different donors. The multiple temperature shifts were compared to optimal storage conditions without temperature shifts. Automated trypan blue dye exclusion and IFN-c ELISpot were used to measure cell viability, recovery, and functionality after cryopreservation in the standardized xeno-free cryomedium IBMT I and cell storage under 3 different conditions. The results were shown to minimize PBMC viability, PBMC recovery and T-cell functionality as measured by IFN-c ELISpot. Hence, temperature fluctuation has been shown to directly affect cell integrity, and the importance of carefully choosing optimal sample storage conditions [6, 14].

2.3 Effects of slow cooling and super cooling on cell viability

Since cooling rate is a major determinant of cell viability following cryopreservation, cryopreserved cells tend to die if it has been linked to intracellular ice formation (IIF) [15]. Research has shown that it has proved beneficial to avoid variable degrees of supercooling in multiple samples by deliberately inducing freezing (nucleation) at a point when the samples have cooled a few degrees below their equilibrium freezing point [16]. Research suggests that cell volume has a pivotal role in the occurrence of IIF than extracellular nucleation temperature or intracellular supercooling. Results indicated that larger cells were more likely to have IIF than smaller cells, and that smaller cells can withstand the supercooling effect before forming intracellular ice [15]. Other research has shown that in post-intracellular freezing, the plasma membrane lost its ability to act as a barrier for extracellular ice, which was similar to the damage caused by osmotic stresses [17].

Optimal slow cooling conditions resulting in retained cell viability are defined by the cooling rate that permits some cell dehydration without the formation of significant amounts of intracellular ice. Tolerances for cell shrinkage and intracellular ice formation vary between cell and tissue types. Ice formation in slowly cooled systems usually begins in the extracellular solution surrounding the biological material. Because ice is pure water, as ice formation occurs, the concentration of solute outside the cells increases and the cells begin to lose water by osmosis resulting in cell

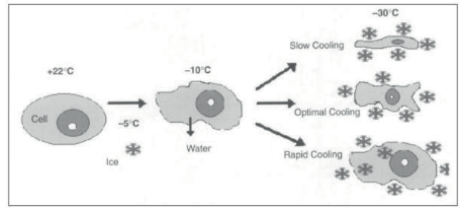

Figure 2.
Different cooling rates showing physical events occurring in cells as they start cooling.

shrinkage. Cooling samples to their freezing point and beyond does not automatically result in freezing the samples at the equilibrium freezing point. Invariably, samples tend to under cool—often referred to as supercooling— to a varying degree that depends on the cooling rate, sample size, presence of nucleating agents, which are foreign particles in a solution that catalyze the formation of an ice nucleus, initiating the freezing process [16]. As samples start the cooling process and as it achieves approximately −5°C, the cells including the surrounding remain unfrozen. Ice starts to form as the temperature drops below −5 down to −15°C. At this stage, the cells and the external medium remain liquefied still, as the plasma membrane blocks the build of ice crystals into the cytoplasm. While the supercooled water in the cells has a greater chemical potential than the water in the partially frozen exterior solution; therefor, water flows out of the cells osmotically and freezes in the external medium. **Figure 2** shows the subsequent events occurring in the cells physically, depending on the cooling rates suggested [18].

Some studies were focused on the observation of the induced IIF which was described earlier in this chapter, which was a result of the water flux across the cells membrane during the freeze–thaw cycle [18]. According to a study model, researchers proposed that IIF may be induced by the plasma membrane through the effects of external ice on the plasma membrane, also known as surface-catalyzed nucleation (SCN), or by the intracellular particles, also known as volume-catalyzed nucleation (VCN). These different effects depend on the freezing conditions used. Also, they suggested that the effects of variables could be minimised if cells were cooled at rapid rates to avoid water flux during the process of freezing [19].

2.4 Method A, B and C

Different cooling methods are commercially available for PBMCs cryopreservation. Method A samples are placed in isopropanol chambers and into −80°C freezers, or into the vapor of liquid nitrogen at a temperature that varies between −135°C and − 190°C. This method is very simple and low in cost, but it does not provide any evidence for traceability or to verify the cooling rate. Therefore, this method would be avoided in clinical settings where a higher assurance of cell recovery and traceability of the freezing process is needed [20]. The freezing of samples using isopropanol filled devices (method A) requires long equilibration times and can introduce variability based on vial position, so the performance is dependent on vial position and continuous isopropanol replenishment. Method A was eventually excluded from the comparison study due to health and safety concerns relating to the use of the Isopropanol. Using programmable freezers (method C) can keep highly reproducible freeze rates, but are also costly, hard to maintain, susceptible to malfunction, and requires large spaces and energy [21]. Method B which is based mainly

on an alcohol-free freezing at the rate of −1°C/minute combined with a − 80°C freezer [22]. This method ensures high thermal control and reproducibility while maintaining a small footprint [21]. A critical factor that influences the survival of cells during cryopreservation is the choice of an optimal cooling rate [20]. Several studies have been done to assess the cell viability for PBMCs, and it was shown to be consistently above 95% before freezing. An assay was blotted to show viability after freezing using either controlled-rate programmable freezer (method C), or the cell-freezing container (method B). In both methods, cells were frozen and stored at −80°C, then further stored at −150°C for 5 days. PBMCs were analyzed via flow cytometer using propidium iodide as a post-thaw viability. The viability rates were shown to be insignificant in the difference between both methods [21]. Nevertheless, the use of method C is thought to minimize two cell damage effects. The first effect, called solution effect which is extensive cell dehydration. The second effect, called mechanical damage which is intracellular ice crystallization. This is further explained as the continuous adjustments of the temperature reduces based on the temperature of the cells, therefore, compensating for fusion heat and reducing of supercooling effects [23]. This temperature compensation is provided by a programmed decrease in chamber temperature that both initiates nucleation and subsequently compensates for the release of the latent heat of fusion. The major variables involved are the rate of chamber temperature decrease, hold temperature and duration and the rate of temperature increase [16].

2.5 Thawing processes

As PBMCs survive cooling to ultra-low temperature, it is still challenging during the thawing processes at which it can exert effects on survival comparable with those of cooling [18]. So, determining how they survive both cooling and subsequent return to physiological condition is the consideration. An important question would be whether they freeze intracellularly or not, which occurs when cooling is too rapid as explained earlier (**Figure 2**). One study has developed equations that describe the kinetics of water loss and predict the likelihood of intracellular freezing as a function of cooling rate. Although it is necessary to avoid intracellular freezing to accomplish survival, but it is not sufficient. Slow freezing can introduce injury to the cell [24], as described earlier in Section 2.3. A study was developed to identify the risk in the addition of ice-chilled washing media to thawed cells at the same temperature, which was shown to be a high-risk practice that yielded significantly lower viability and functionality of recovered PBMC. This study also compared the previously mentioned outcome to the use of warm cryovials in temperatures of 37°C while adding a warm washing medium. The thawed PBMC in cryovials were kept up to 30minutes at 37°C in the presence of DMSO, and surprisingly showed that exposure to DMSO was a low-risk practice during the thawing process [25].

2.6 Factors that impact PBMCs stability

A major reason to use a freezing equipment or protocol rather than simply placing samples in cold environments is that the temperature compensation provided during controlled rate preservation for release of the latent heat results in improved post-cryopreservation cell viability [16]. In addition, research has focused on improving the interaction between cooling rates and the permeability of the plasma membrane to water and cryoprotectants [26]. The addressed interaction plays a

major role in PBMC stability. So, as the biological metabolism in cells dramatically diminishes at low temperatures, research has shown that cells were unable to endure the low temperatures. However, it is in fact the lethality of an intermediate zone of temperature (−15 to −60°C) that cells must traverse twice—once during cooling and once during warming [18]. Research studies recommend the induction of on-site training that facilitate a standardized method for cell counting, freezing, and thawing in order to maintain an environment with reduced variation in cell recovery. Nevertheless, external quality control programs can also enable the optimization of viability and cell recoveries with higher yields and viability to maximize the value of PBMC to be collected and stored for research studies [12].

3. Materials and methods

3.1 Study sample preparation

A total of 12 blood samples were collected and prepared from healthy adults. These samples were collected and processed according to Qatar Biobank (QBB) procedures. CPT closed sample collection kits with tubes containing additives of sodium citrate were used to collect whole blood [27]. A total of 24 viable cells in 1ml aliquot tubes were divided; every aliquot from the same parent CPT tube was placed in method B and method C, simultaneously. To obtain accurate measurements, viable cells were stored for a minimum of 24 hours in liquid nitrogen vapour.

3.2 PBMC isolation and cryopreservation

The procedure of PBMC separation was carried out in the laminar flow cabinet, which was turned on for at least 10 minutes before the work was started. The surface was disinfected using sodium hypochlorite followed by 70% ethanol and then type 1 water. CPT tubes were processed following a standard protocol [11] After centrifugation, the tubes appeared to have layers as shown previously in **Figure 1**. PBMCs were transferred using sterile tips into 15 ml sterile prelabelled intermediate tubes. This intermediate tube was connected to a parent tube by a Laboratory Information Management System tool for our labs. 100μl of PBMC were sub aliquoted from the intermediate tube into 5 ml prelabelled plain tubes that were also connected to the parent tube. The plain tube was processed in the cell counter to check the number of WBCs before running the samples in the flow cytometer (Section 3.4). In the 15 ml sterile intermediate tube, sterile phosphate-buffered saline (PBS) was added (in laminar flow cabinet) till 15 ml as first wash cycle. The cells were mixed gently by inverting the intermediate tube 5 times, then it was centrifuged for 15 min at 300 RCF at room temperature. The supernatant was disposed in an empty sterile waste bottle. The cell pellet was resuspended by gently vortexing or tapping tube with index finger. Sterile PBS solution was added until the 10 ml mark as second wash cycle. The tube was mixed by inverting 5 times, then it was centrifuged for 10 min at 300 RCF at room temperature. The supernatant was disposed in an empty sterile waste bottle. 1 ml of 10% DMSO was added to the tube and gently pipetted to mix with the cell suspension. 2 aliquots were created from each one parent tube in the corresponding sterile 1 ml tube. Using our laboratory information management system the parent tube was barcoded and scanned to be linked to 2 aliquots each. The aliquots were placed on a cooling shell to allow the cryoprotectant to enter the cells, and to prevent

heat generation that can damage the cells. Keeping the specific timeline to allow stabilization, which is between 20 to 30 minutes to prevent the toxic effects of DMSO on the cells. 12 out of 24 created aliquots were transferred to method C, where they were gradually cooled in a user pre-defined temperature in the controlled rate freezer that is 1°C per 1 minute until −30°C, after that cooling rate is increased up to 5°C per 1 minute until −100°C is achieved, to ensure that the freezing process runs gradually to keep the cells, membranes, and cellular organelles safe and intact. Eventually, these aliquots were then stored in liquid nitrogen vapour. The remaining 12 created aliquots were transferred to method B, which uses a fridge temperature pre-cooled cool cell box, in which samples were then transferred to be cryopreserved within 4–24 hours of cool cell use time in the -80C freezer. This has been verified with an internally validated method in parallel with method C, in any contingency situation with the goal to cryopreserve high-quality quality PBMC samples.

3.3 Thawing the PBMCs

The cryopreserved PBMCs were retrieved from vapor phase of liquid nitrogen storage and placed directly in -80C portable freezer until samples were thawed. A standard thawing procedure for PBMCs was followed [28]. After thawing, cells were resuspended in PBS buffer as a preparation step for flow cytometer cell viability analysis explained in the next section. A standard thawing procedure is equally as or perhaps more important for obtaining maximum viability and recoveries of cryopreserved PBMC. The thawing procedure should also become part of the validation exercise to ensure reproducible sample preparation and cryopreservation.

3.4 Assessing the cell viability using flow cytometry

Prior to processing samples in the flow cytometer, a cleanse panel was run followed by a fluorescent microspheres suspension check. This step is mandatory as a routine quality control check prior to daily instrument operation. The PBMCs were extracted from the processing of CPT tubes that was previously explained in both sections 3.3 and 3.4. The cells were washed with 400 ul of PBS and centrifuged for 5 minutes at 500xg at 4°C. The supernatant was then discarded, and dyes were added to the cell suspension each prepared as follows, 10 μL of Annexin V-FITC, 20μL of 7-AAD viability dye and 10 μL CD45-APC750. The samples were mixed gently and kept for incubation in the fridge in the dark for 15 minutes. After incubation, 400 ul of binding buffer was added to each sample. Finally, the results were checked for the acceptable viability percentages for each sample as shown in **Figure 3**.

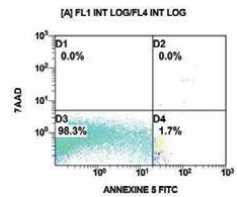

Figure 3.
Dot plot diagram from PBMCs showing cell population. D3. Double negative (Annexin V and 7-AAD negative) healthy cells. D4. Annexin V positive, 7-AAD negative apoptotic cell. D2. Annexin V & 7-AAD double positive necrotic cell.

3.5 Statistical analysis

The temperature for method B was recorded at 10-second intervals over a 4-hour period, which was repeated twice. Also, the temperature for method C was recorded over 2-hour period. Thermocouple probes were calibrated and set up with a temperature data logger for method B to record the temperature every 10 seconds using temperature record data-logger software, while instrument-specific temperature record software was used to generate temperature curves for method C, in addition to, temperature record data-logger. Additionally, Student's t-test was generated based on the comparison of standard deviation and mean values of both method B and method C.

4. Results and discussion

4.1 Comparison between method B and method C -cooling rate temperatures

Following manufacturer specification, method B had a cooling rate of 1°C per 1 minute. This fact was verified using probes as mentioned in Section 3.5, and the overall average rate was 0.98°C with slight differences in cooling rate from −15.5°C to −30°C, and from −30°C to −50°C, as shown in **Table 1** and temperature curve is shown in **Figure 4**. Cooling performance of method C was measured by instrument-specific temperature record software configured with the instrument as shown in **Table 2** and **Figure 5**, to detect and record all temperatures and curves, in addition to temperature record data-logger as shown in **Table 3** and **Figure 6**.

Method B, temperature ranges	Cooling rate (°C)
Average rate per 1 min for 4 h	0.98
From 15.5°C to - 30°C	1.33
From −30°C to −50°C	0.71

Table 1.
Method B temperature recorded over 4-hour interval.

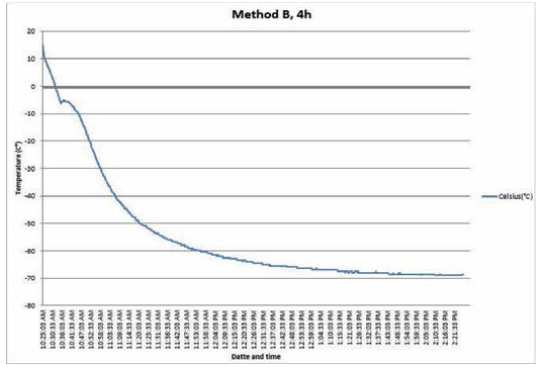

Figure 4.
Method B temperature curve for 4-hour interval.

No.	Temperature (°C)	Duration (min)
1	4	8
2	0	1
30	5	
4	−100	100

Table 2.
Method C freezing cycle program in instrument-specific temperature record.

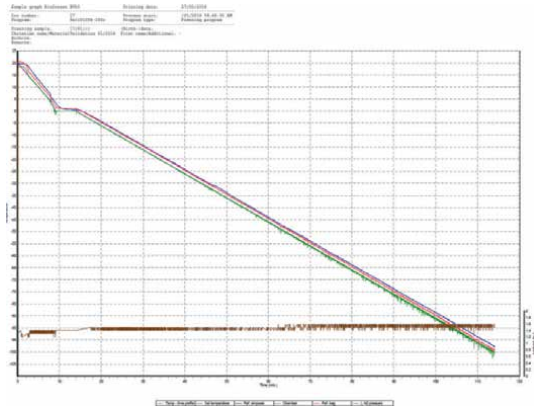

Figure 5.
Curve of method C freezing cycle program in instrument-specific temperature record.

Method C, temperature ranges	Cooling rate (°C)
Average rate per 1 min for 2 h	0.93
From 15.5 °C to – 30°C	1.08
from – 30°C to – 100°C	0.79

Table 3.
Method C temperature recorded over 2-hour interval.

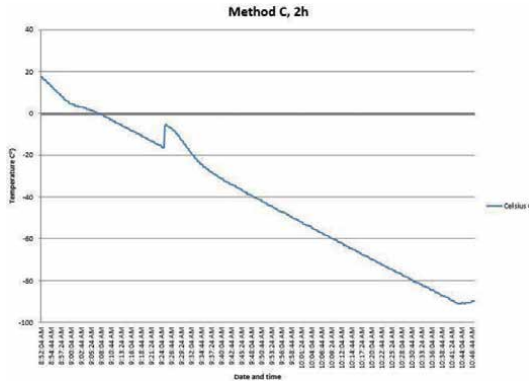

Figure 6.
Method C temperature curve for 2-hour interval, using temperature record data-logger.

As shown in **Figure 4**, latent heat was generated during freezing, an exothermic process, or heat release, known as the latent heat of fusion or crystallization, during ice formation must be conducted away from the material being frozen.

Differences in temperature rate between method B and method C were not statistically significant as shown in **Table 4** below.

4.2 Adjusting the cooling rate for better stability of PBMCs

A critical variable factor that is the main scope of this chapter is the effect on the survival rate of cells by the cooling rate. During slow cooling the cells are exposed to DMSO, which is harmful for cell viability, and the concentration of external liquid is increasing, leading to dehydration as a consequence of efflux of water from cells due to change in osmotic status. Therefore, cells can shrink and become deformed. Using fast cooling rate, the dynamic characteristic of the cell membrane must be considered. The intracellular water cannot pass through membrane fast enough and freezes inside the cell. This process is lethal for the cell. During freezing, the following phases are appearing in the cell media: liquid phase cooling 1°C/min, supercooling (undercooling) is happening below the freezing point of media (DMSO freezing point is −5°C), this phase is followed by thermal increase as its exothermal reaction. Phase change-liquid to solid phase change followed immediately after supercooling. Solid phase I freezing is same 1C°/min. End solid phase I freezing is usually between −25°C to −50°C. Protocol of freezing can be shortened in the duration of the reaching the solid phase freezing, and cooling rate can be increased 5°C/min to the end of solid phase II freezing, which is between −80 and − 90°C. This provides adequate temperature security by preventing sample warming above End solid phase I. As the optimal cooling rate is essential for cell viability during cryopreservation, and specifically for that purpose, 4 control rate protocols were developed and compared in terms of temperature stability and viability. First program was following the control rate according to method B protocol with 1°C per 1 minute. Second program was designed to have cooling rate 1°C per 1 minute until −30°C, after that, cooling rate was increased up to 5°C per 1 minute until −100°C is achieved, as shown in **Figure 6** and **Table 5**.

A third programme was created to prevent the impact on cells by latent heat generation, which is clearly shown in **Figure 7** below.

		Method B	**Method C**
1 °C/1 min	Mean	0.98	0.93
	St dev	0.47	1.83
	N	200	200
	Sp2	198	14.07
	t calc	0.0036	
	t value from t table,	2.62	
	CI 99%		
	ItI < It tabI	0.003 < 2.62	

Table 4.
Comparison between method B and method C, program of 1°C/1 min.

No.	Temperature (°C)	Duration (min)
1	4	8
2	0	1
3	0	5
4	−30	6
5	−100	14

Table 5.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

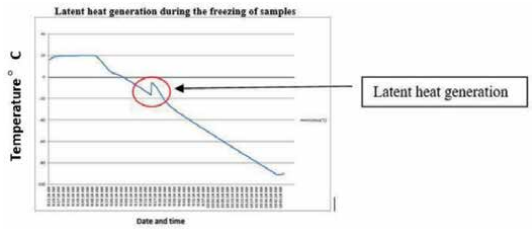

Figure 7.
Latent heat generation during the freezing of samples.

To improve the viability of the cells, an additional plot between −12°C and − 30°C was added (4°C/min), as shown in **Table 6** below.

The shape of the peak for latent heat generation in program 4 as shown in **Figures 8** and **9** was not as sharp as at the first program. Both of the programs were reaching the point of freezing media −5°C. And cooling rate was increasing for 4°C per minute to prevent the supercooling. To keep the cooling rate during crystallization as close to 1–2°C/min, a much greater difference between gas and sample had to be maintained as illustrated in **Table 7**. Heat of fusion was transferred through the wall of ampoule and heat capacity of the sample. After the intracellular phase transition was done (at −30°C), cooling rate can be increased to 5°C per minute.

The next improvement step was done at the temperature level of −30°C to achieve equal temperatures between the chamber and reference ampule (equal to sample) before increasing the cooling rate, as shown in **Table 8** below.

No.	Temperature (°C)	Duration (min)
1	0	5
2	−12	12
3	−20	2
4	−30	10
5	−100	14

Table 6.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

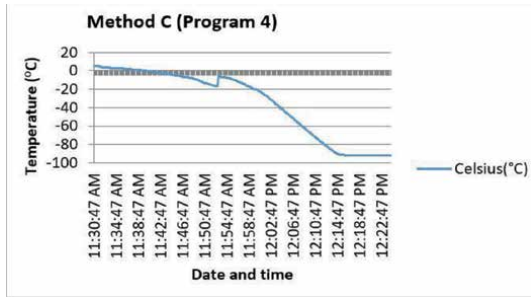

Figure 8.
Method C temperature curve for program 4, using temperature record data-logger.

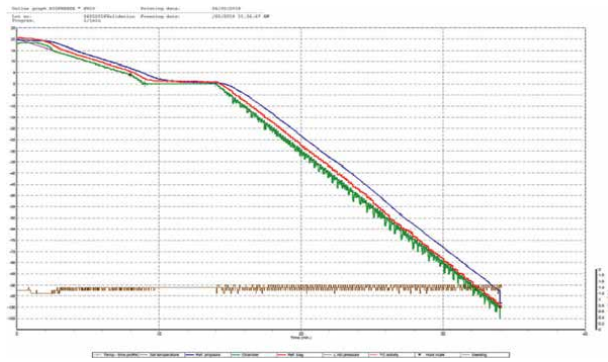

Figure 9.
Curve of method C freezing cycle program in instrument-specific temperature record using program 4.

Program	Temperature	Average difference between chamber and reference probe in ampule	Average difference between reference probe in ampule and set temperature	Parameter criteria
1 °C/1 min	16–0 °C	3.83	1	3
	0 °C to –100°C	1.99	2	
5 °C/1 min	16–0 °C	2.7	1.6	
	0 °C to –100°C	4.9	1.8	
5 °C/1 min [4 °C]	16–0 °C	2.7	1.5	
	0 °C to –100°C	3.4	1.32	

Table 7.
Difference between chamber and reference probe in ampule, and difference between reference probe in ampule and set temperature with regards to different programs.

The instrument-specific temperature recording software has an option to define the ΔT between the set value and the actual value. If ΔT becomes smaller value, the program continues automatically. This is to assure that sample temperature has been stabilized at the chamber temperature before it is cooled down with the defined freezing rates of the freezing program.

No.	Temperature (°C)	Duration (min)		Heat	Hold
1	**0**	**5**	**5**		
2	−12	12	17		
3	−20	2	19		
4	−30	10	29		Stop
5	−100	14	43		

Table 8.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

4.3 PBMCs viability using method B and method C (comparative study)

Viability was measured and compared before and after thawing [28]. Lowest result obtained was 95.9% and highest was 98.9% and the difference between each was between 0 and 1% which is in the acceptable range, shown in **Table 9**:

Then, method C protocol with first program that had the same cooling rate as in method B, with 1°C per 1 minute. Viability was measured and compared before and after thawing [28]. Lowest result obtained was 95.6% and the highest as 98.4% and difference between each was between 0 and 1.5% which is within an acceptable range, as shown in **Table 10** below:

Viability was measured based on the difference between pre-thaw and post-thaw viability percentages that were obtained from the flowcytometry. Viability 80% and 75% recoveries are recommended [28], and both methods are within acceptable ranges.

The initial hypothesis was that difference between method B and method C in terms of viability will not be statistically significant, which was proven by Student t test, as shown in **Table 11**. Statistical significance during this comparative study

Sample # in Method B	Pre-thaw Viability %	Post-thaw Viability %	Difference %
TUR0014631	96.7	96	0.7
TUR0014632	98.5	96.7	1.8
TUR0014633	97.3	96.8	0.5
TUR0014634	96.7	97.1	0.4
TUR0014635	97.5	96.5	1
TUR0014636	96.3	96.3	0
TUR0014637	99.9	98.9	1
TUR0014638	97.5	97.8	0.3
TUR0014639	97.2	97.1	0.1
TUR0014640	98.2	98.3	0.1
TUR0014621	97.5	97.4	0.1
TUR0014622	96.4	95.9	0.5

Table 9.
Method B viability overview.

Sample # in Method C (Program 1)	Pre-thaw Viability %	Post thaw Viability %	Difference %
TUR0014631	96.7	96.2	0.5
TUR0014632	98.5	97	1.5
TUR0014633	97.3	96.8	0.5
TUR0014634	96.7	96.7	0
TUR0014635	96.2	97.2	1
TUR0014636	96.3	95.6	0.7
TUR0014637	99.9	98.4	1.5
TUR0014638	97.5	97.8	0.3
TUR0014639	97.2	97.1	0.1
TUR0014640	98.2	98	0.2
TUR0014621	97.5	97.4	0.1
TUR0014622	96.4	96.4	0

Table 10.
Method C viability overview.

Sample No.	Parent tube sample #	Viability % in Method B	Viability % in Method C
1	TUR0014631	96	96.2
2	TUR0014632	96.7	97
3	TUR0014633	96.8	96.8
4	TUR0014634	97.1	96.7
5	TUR0014635	97.5	97.2
6	TUR0014636	96.3	95.6
7	TUR0014637	98.9	98.4
8	TUR0014638	97.8	97.8
9	TUR0014639	97.1	97.1
10	TUR0014640	98.3	98
11	TUR0014621	97.4	97.4
12	TUR0014622	95.9	96.4
	Mean	97.2	97.1
	St dev	0.86	0.76
	N	12	12
	Sp2	11.2	3.3
	t calc	0.03	
	t value from t table for 34 df,	3.06	
	CI 99%		

Table 11.
Comparison between method B and method C, viability percentage.

was calculated using t test for temperature protocols (cooling rate variation): ItI < It tabI 0.003˂2.62 and post thaw viability detected on flow cytometer ItI < It tabI 0.03 < 3.06. Expected accuracy for post thaw viability interval was ±20% and obtained post thaw viability was 0.55%. Precision-reproducibility estimation during 5 different days was post thaw viability 0.66% when the acceptable interval was ±20%.

Precision estimation was designed through 4 days, 6 samples every day, for a total of 24 results from 12 CPT tubes. Between day variation was expected to be low as samples were in a stable frozen state. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the reproducibility conditions. Statistical calculations were made in Microsoft Excel -Tools> Data Analysis> ANOVA, one factor test ordering Sw interserial standard deviation, and using Microsoft Excel functions to calculate Six, Sb and Stott. Results obtained are shown in **Table 12** below. Coefficient of variation (%) (Stot*100/Xsr) was shown to be 0.79%. Hence, results of precision met the requested acceptable criteria. Viability percentages and difference in pre and post thaw viability was in acceptable criteria, lowest was 0.3 and highest was 4.1, as shown in **Figures 10** and **11**, respectively. **Table 13** illustrates all the validation study parameters that were taken and calculated.

Average	**Xsr**	**95.23**
St dev of average of groups	Sx	0.53
	Sx2	0.29
	Sw2	0.57
	Sb2	0
Total st dev	Stot	0.76
Source of Variation Within Groups	Sw	0.76
Source of Variation Between Groups	Sb	0
Coefficient of variation (%) (Stot*100/Xsr)	CV[%]	0.79

Table 12.
CV % calculated.

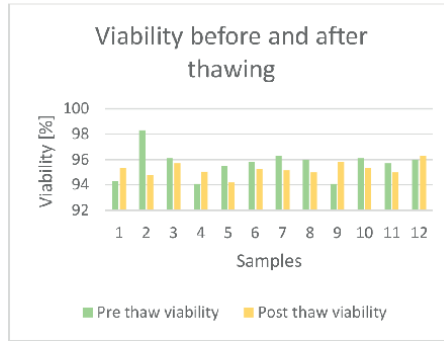

Figure 10.
Pre-thaw and post-thaw viability percentages.

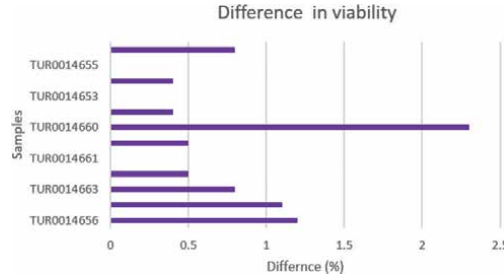

Figure 11.
Pre-thaw and post-thaw viability differences.

Parameter	Required	Obtained	Acceptance	Improvement action
Comparison	T test	Temperature protocols: ItI < It tabI 0.003 < 2.62	pass	
		Viability ItI < It tabI 0.03 < 3.06		
Sensitivity	1 °C/min	1 °C/min	pass	
		5 °C/min (from-30°C)		
		5 °C/min (from −20 °C 4 °C/min)		
Accuracy	Temperature interval ± 3C	Temperature interval < 1.5 °C Post thaw Viability interval 0.55%	pass	
	Post thaw Viability interval ± 20%			
Precision	Temperature interval ± 3C	Temperature interval < 1.5 °C	pass	
	Post thaw Viability interval ± 20%	Post thaw Viability interval 0.66%		

Table 13.
Showing all values obtained through the validation study.

5. Conclusions

The presented study showed that there was no statistically significant difference in cooling methods. However, advantage of Method C is demonstrated in a major decrease in cooling time by reducing the PBMCs processing life cycle, without a need for intermediate storage space while sample traceability is enhanced by using the device software which can be integrated with our LIMS system. The risk of human error, which might occur with Method B, is minimized by reducing operator intervention. Both methods can be used in accordance with laboratory preferences, budget, and guidelines with integrated risk assessments and instrument downtime contingency plans.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

Plant Cryopreservation Importance, Approaches and Future Trends

Victor Acheampong Amankwaah, Ruth Naa Ashiokai Prempeh and Marian Dorcas Quain

Abstract

Plant cryopreservation is useful for long term storage of clonal germplasm and endangered species. Clonally propagated crops which produce recalcitrant seeds cannot be easily conserved using conventional methods. Preservation of plants *in vitro* is limited to two years and not ideal for germplasm storage for a very long time. The need to conserve plant genetic resources through cryopreservation techniques to mitigate the effects of climate change such as extinction of certain plant species cannot be underestimated. Different cryopreservation methods including dehydration, programmed freezing, vitrification and v cryo-plate are employed in the long-term storage of different plants. These methods are usually based on the principle of the removal of freezable water from tissues by physical or osmotic dehydration followed by ultra-rapid freezing. There have been several advancements in the identification and use of cryoprotective agents, nonetheless, its toxicity remains a challenge. To accelerate plant cryopreservation, there is the need for the development of global expertise. The current practice for the conservation of germplasm in the Biotechnology Laboratory in Ghana is through the use of slow growth media. Moving forward, there is the need to work on developing cryopreservation protocols for preservation of germplasm using liquid nitrogen and cryogenic refrigerators.

Keywords: cryopreservation, vitrification, conservation, gene bank, shoot tip

1. Introduction

As early as 2000 BC, archaeological findings has shown that icehouse were used throughout Mesopotamia to store foods [1]. Since time immemorial, the preservation of biological material has been known. The storage of biological material at ultra-low temperatures is referred to as cryopreservation. In broad terms, cryopreservation refers to the study of life at low temperatures [2]. Plant cryopreservation is a conservation method that permits long-term storage of tissue samples at very low temperatures of −135°C to -196°C with little risk of causing variation. Cells can successfully be cryopreserved in liquid nitrogen when extracellular water has been removed to the extent that any remaining water form the so-called biological glass

(vitrification), thereby mitigating the adverse effect of ice crystal formation and growth [3]. Cryopreservation for storage of plant cells, tissues, and organs became operational in the 1960s till date. Long term storage of *in vitro* cultures of secondary metabolite cell cultures, embryogenic cultures, clonal germplasm, endangered species, and transgenic products remains a sine qua non for many scientists, organizations and companies [4]. In the case of clonally propagated crops which produce recalcitrant seeds and cannot be readily conserved by conventional methods through seed preservation, cryopreservation is important for long term conservation. Over the years, a lot of research on different crops to study the feasibility of the long-term storage of plant species has taken place. Prof. Akira Sakai, researched on mulberry twigs after exposing them to liquid nitrogen. This study is reported to be the pioneer in plant cryopreservation research [2, 5]. Research in cryopreservation in the twentieth century was devoted to basic studies of ice formation, vitrification of solutions and the beginnings of cryopreservation as a long-term storage technique [4]. In recent times, cryopreservation research has focused on practical procedures for gene bank storage, thereby enabling cells and meristems to be cryopreserved by direct transfer into liquid nitrogen. The development of simple and reliable methods for cryopreservation has led to cryo-banking [6].

2. Importance of cryopreservation of plants

A prerequisite for the short- and long-term survival of plant species in their natural habitat is genetic diversity [7]. Biological diversity conservation importance was recognized in 196 countries this led to the generation of a treaty that includes the sustainable use of its components, fair and equitable participation in the benefits derived from the use of plant resources [3]. The long-term conservation of tissues using cryopreservation has been increasingly used in recent years as it requires very little storage space, minimal upkeep, and eliminates the risk of contamination, makes the germplasm available for posterity, and its applicability to a wide range of plant tissues [6, 8].

2.1 Advantages of cryopreservation over other methods

Plant genetic resources are usually conserved in their natural habit (*in situ*) or other sites (*ex situ*). Preservation off site is partially used or for the entire population when preservation *in situ* is extremely challenging usually as a result of lack of complete control over many factors that influence the survival of plant materials and its genetic make-up [3]. Maintenance of plant genetic material *in vitro* is more efficient and secure than conservation in the field. *In vitro* conservation has been reported for the long-term conservation of germplasm for approximately two years without sub-culture such as in the case of potato. This notwithstanding, *in vitro* preservation is not ideal for long term germplasm conservation because it is labour consuming, costly, and carries risks of losing germplasm due to human error, such as contamination and mislabeling during sub-culturing [9]. Furthermore, erratic power supply, malfunctions in air-conditioning and lighting system could sometimes pose a challenge for *in vitro* conservation. Moreover, another setback of tissue culture for long term conservation is the induction of genetic variation or somaclonal variation during prolonged sub-culture [6]. Besides, mites, thrips, and other small arthropods can cause extensive fungal contaminations in tissue culture and are difficult to eliminate.

In addition, tissue culture collections are constrained by the occurrence of cellular aging and senescence during prolonged cultivation. The effect of cellular aging may appear in parallel with slow growing endophytic microbes that can accumulate over time [10].

The only *ex situ* conservation method that allows long term survival of organisms at very low temperature and using reagents such as liquid nitrogen is cryopreservation. Plant materials stored in liquid nitrogen have indefinite lifespan in spite of the fact that no biological specimen is immortal [11]. Again, it is the only *ex situ* conservation method used for long-term conservation of plant materials that cannot be stored in seed banks, for instance clonal crops or species with a low number of progenies or recalcitrant seeds. Furthermore, it requires only a minimum space and maintenance efforts (**Figure 1a** and **b**). It has become a very important tool for the long-term storage of plant genetic material [11, 12]. Moreover, in addition to its use for the conservation of genetic resources, cryopreservation can also be applied for the safe storage of plant tissues with specific characteristics. Plant cells of different types, gametic cells, tissues and organs can be cryopreserved [13]. Due to the totipotency, various plant cells can be manipulated to enhance regrowth after cryopreservation, paying attention to genetic integrity.

2.2 Food security, biotechnology and breeding

The world's most important food crops for food, nutrition, and livelihoods most especially for the poorest people are vegetatively propagated crops. Examples of some of these crops include banana (*Musa sapientum*), plantain (*Musa paradisiaca*), sweetpotato (*Ipomoea batatas*), cassava (*Manihot esculenta*), yam (*Dioscorea* spp.), citrus (*Citrus* spp.) and coconut (*Cocos nucifera*) [14]. Plant genetic resources constitute the store of genome information and are important for world food security, crop improvement and conservation of genetic diversity [15]. It is important in breeding programs to obtain new or more productive plants that are resistant to biotic and abiotic stresses, due to the changing weather patterns [2]. Globally, food, feed and fiber utilization are restricted to very few species, hence, advanced biotechnology

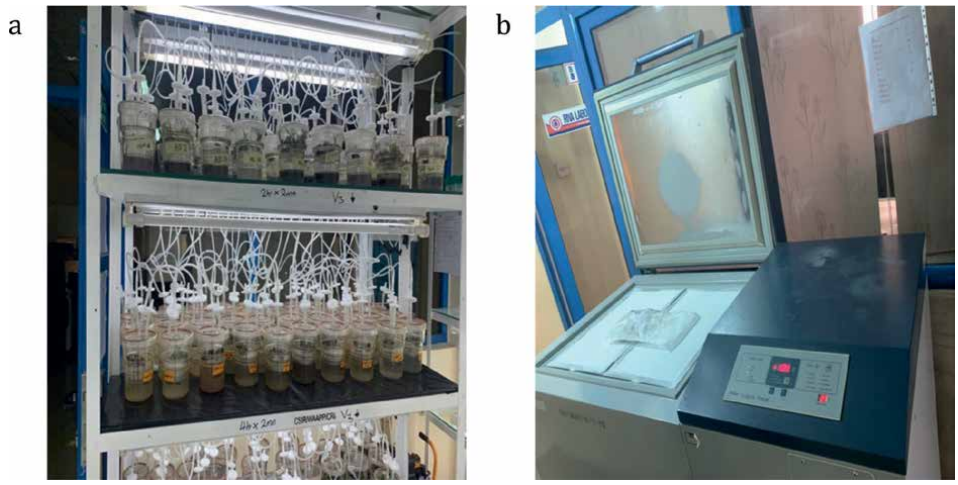

Figure 1.
Comparison of conservation of 150 plantain accessions on (a) RITA temporary immersion bioreactor system compared with the use of a (b) cryo-freezer in terms of space for storage at CSIR-CRI, Kumasi-Ghana.

techniques such as cryopreservation represents an efficient alternative method for *ex situ* conservation of germplasm of various crop species. It helps in overcoming several challenges of storage by conventional means. In recent times over 10,000 accessions through initial *in vitro* introduction and subsequent preservation using cryogenic methods have been used for several crops. Above 80% of these crops belong to crops that are widely consumed such as potato, cassava, bananas, mulberry and garlic [16].

In modern breeding programs, cryopreservation is important for providing long-term storage and international access to various genetic materials. The genetic materials accessible internationally include seeds, pollen and meristematic apices and buds. Plant breeders and horticulturists involved in fruit and forest tree improvement are very much particular about pollen storage. Techniques for pollen culture have been used for decades to obtain haploids or homozygous diploid plants from various plant species such as maize and rice. Regular supply of viable pollen provided by pollen banks takes away seasonal, geographical or physiological limitations of hybridization programs and supports hybrid development between genera and species. Large field areas are required by traditional pollen banks at different stages to synchronize flowering both of which are very labour intensive and needs a lot of funds to be operational. Other methods of pollen banking for the purposes of breeding for short-term storage such as freeze drying, freeze storage, vacuum drying and cold storage in organic solvents lead to frequently observed sharp reduction in pollen viability. The most efficient means of pollen storage is cryopreservation that does not require any expensive cryostats. This is so because pollen grains can be directly immersed in liquid nitrogen for long-term storage [16].

Advanced biotechnology application such as cryopreservation is a very good efficient method for *ex-situ* conservation of plant germplasm. This method supersedes the challenges and limitations of conventional methods seed banks and conventional orchards [2]. Preservation of plant germplasm for plant breeding and biotechnology has long been recognized and it is very essential for enhancing breeding activities. It has been reported that easy access to diverse plant germplasm is a pre-requisite for breeding more productive cultivars. This in the long run ensures food security [16, 17]. With respect to biotechnological interventions, the consistently evolving area of phyto-chemical production via biotechnological methods is supported by cryo-banking of root cultures, embryogenic and non-embryogenic cell lines to ensure their genetic and biochemical stability [16, 18].

2.3 Agrobiodiversity

Plants are recognized as a vital component of biodiverse ecosystems (the carbon cycle, food production and bio-economy) [19]. An important issue concerning human population worldwide is the conservation of plant biodiversity. Plant biodiversity is a natural source of products to the food industries. Provision of basic raw materials is its hallmark. Maintenance of plant biodiversity in their natural habitat, as well as domesticated and cultivated species on the farm or in the surroundings where they have developed their distinctive characteristics, represent the *in situ* strategies. Due to heavy loss of species, populations and ecosystem composition leading to loss of biodiversity, *ex situ* conservation is a viable way for saving plants from extinction, and in some instances, it is the only possible strategy to conserve certain species [17]. Plant genetic resources are highly important for agro-biodiversity because they can be used to breed new or more productive crops that can withstand biological and environmental stresses [12, 13].

2.4 Cryotherapy for virus elimination

Systemic pathogens such as viruses, phytoplasmas and bacteria could be eliminated by treating shoot tips with liquid nitrogen using cryopreservation protocols. It is a novel approach for pathogen eradication in plants. The uneven distribution of viruses and other pathogens in shoot tips allows the elimination of the infected cells by injuring them with the cryo-treatment and regeneration of healthy shoots from surviving pathogen-free meristematic cells. Cryopreservation methods have been useful in pathogen eradication by means of shoot tips cryotherapy [17]. The use of cryotherapy to remove viruses from vegetatively propagated crops has been reported [4]. It allows treatment of large numbers of samples and results in a high frequency of pathogen-free regenerants. Consequently, it has the potential to replace more traditional methods like meristem culture, chemo- and thermo-therapies. This method has been utilized for eradication of severe pathogens in banana, citrus, grapevine, raspberry, potato and sweetpotato [17].

2.5 Importance of cryopreservation in the era of climate change

Greater risks of extreme weather and changes in climate variables such as prolonged drought and storms are events that biomes will have to adapt as one of the measures to prevent extinction [11, 20]. Effects of climate change on biodiversity, agricultural production and food security have been a matter of great concern [21]. The need to adopt strategies to conserve plant genetic resources to mitigate the effects of climate change that has a potential of causing the extinction of certain plant species cannot be underestimated. One strategy to address the issues of climate change in order not to lose endangered species is cryopreservation. For instance, critically endangered species growing in the wild in Finland has been successfully cryopreserved to enable its long-term conservation through the use of droplet vitrification protocol. Additionally, protocol development for cryogenic preservation of plant species is an additional tool to *ex situ* conservation toolbox for the maintenance of plants to avert the effects of climate change [11].

3. Stages in cryopreservation

Depending on the selected technique, cryopreservation is made up of different stages which includes preparation and explant excision, preculture, cryoprotection, vitrification/dehydration, fast cooling in liquid nitrogen, rewarming, cryoprotector elimination, regeneration and plant culture [8, 22].

In an effort to preserve biological materials for cryopreservation, the following steps are followed. The first step involves harvesting or selection of material, the growth stage has to be considered where applicable. Much attention should be paid to volume or size, density, pH and morphology. The second stage has to do with addition of cryo-protectant agents that include glycerol, salts, sugars, glycols that are added to samples. This stage is then followed by the application of different methods of freezing to protect cells from damage and cell death by their exposure to the warm solutions of cryoprotective agents (CPA). After all these have taken place, the cryopreserved samples are stored in −80°C in a freezer for at least 24 hours before transferring it to storage vessels. Finally, the process of thawing is initiated which involves warming the biological samples in order to control the rate of cooling and prevention of cell damage caused by crystallization [23].

4. Plant material used for cryopreservation and cryopreservation agents

The state of mother plant especially with regards to physiological state is a key factor for the success of cryopreservation. For cryopreservation techniques, any totipotent tissue may be used. Most commonly used tissues are shoot tips, and to a lesser extent, somatic embryos and embryonic axes. Shoot tips and somatic embryos for cryopreservation require tissue culture systems with established micropropagation regimes [24].

Decisions concerning the choice of a plant material for cryopreservation are dependent on plant type and reason for storage. Based on knowledge of plant vulnerability, curators need to make decisions on which plants to store based on their knowledge of plant vulnerability. The decision to select a plant part for cryopreservation technique depends on growth conditions. Generally, practice shows that plants that are diseased or not thriving for any reason are generally poor candidates for cryopreservation. Plant materials should be in an optimal growth phase, dormant materials should fully break dormancy, and where appropriate fully cold acclimated [25]. The question thus remains about how amenable plants indigenous to the tropical regions can respond successfully to cryopreservation.

Meristems and embryos are explants normally conserved using encapsulation techniques. Alginate beads which contain mineral salts and organic substances are used for the encapsulation of meristems and embryos. Cryopreservation agents are used for the treatment of plant genetic material as in the case of vitrification methods. The most commonly applied vitrification solutions include vitrification solution number 2, which contains glycerol, ethylene glycol and sucrose. These reagents are used by synseeds during regrowth so that they quickly grow to prevent loss of viability. Vitrification solutions contain penetrating and non-penetrating cryoprotective substances to preserve both inside and outside of plant genetic material and prevent the formation of lethal ice crystal so that cells remain viable for a long period of time [26].

Pollens are cryopreserved for breeding purposes. Viability of pollen after cryopreservation depends on a number of factors. Pollen moisture content, freezing and thawing procedure, physiological stage of mother plant, flowering stage, plant vigor and genotypic differences are the factors that determine pollen viability [16, 24].

5. Methods of cryopreservation and application

Different techniques are employed in the long-term storage of different plants. These techniques include dehydration, controlled-rate cooling and vitrification [4]. Cryopreservation technique is based on the principle of the removal of freezable water from tissues by physical or osmotic dehydration followed by ultra-rapid freezing. In cryopreservation procedures, water plays a central role in preventing freezing injury and in maintaining post-thaw viability of cryopreserved cells stored in a small volume, requiring a very limited maintenance. Classical freezing procedures encapsulates the use of different cryoprotective solutions combined with pre-growth of material followed by slow cooling (0.5–2.0°C/min) to a determined pre-freezing temperature (usually around −40°C), rapid immersion of samples in liquid nitrogen, storage, rapid thawing and recovery [17]. Cells with low water content which includes pollen, seeds, and dormant tissues of stress-tolerant species, may be introduced to low temperatures such as in the case of using liquid nitrogen without lethal damage. On the other hand, plant cells with higher water content present considerable problem as a result of ice crystal growth causing cell bursting [3].

For any cryopreservation to be successful, it is important to avoid the lethal intracellular freezing that occurs during rapid cooling. Consequently, in any cryogenic procedure, the cells and shoot tips must be sufficiently dehydrated in order to preclude freezing and to allow vitrification in liquid nitrogen [12].

In the past 25 years, many cryopreservation techniques have been established based on the conventional slow freezing techniques. The different approaches used include vitrification, droplet vitrification, dehydration and pre-growth and pre-growth dehydration [19]. Cryopreservation methods are commonly used globally. It has been reported that new cryogenic methods using cryo-plates (the V cryo-plate and D cryo-plate) are advantageous over early developed methods. Advantages are manifested in ease of handling during the procedure and high regrowth rates after cryopreservation [12].

5.1 Dimethyl sulfoxide droplet

Since 1866, dimethyl sulfoxide (DMSO) has been commonly used for the cryopreservation of tissues because of its low cost and relatively low level of cytotoxicity [27]. DMSO acts by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature. With this method, plant materials are treated with a 10% DMSO in liquid Murashige Skoog (MS) medium with 30 g sucrose, 0.5 mg/l zeatin riboside, 0.2 mg/l GA3 and 0.5 mg/l IAA. This method appears to be simple because only 10% DMSO in liquid medium is used as cryoprotectant solution. The explants (shoot tips of 2–3 mm) are then incubated in MSTo medium overnight at 22°C and treated with cryoprotectant solution (10% DMSO in MSTo medium) for 1–3 h at RT followed by transfer into droplets of 2.5 μl cryoprotectant solution one by one on aluminium foil. The aluminium foil is then immersed directly into cryotube filled with liquid nitrogen [6]. DMSO droplet has been routinely used for by the for safe and long-term conservation storage of shoot tips of sweetpotato by the International Potato Center (CIP) [9].

5.2 Dehydration

It involves dehydration of samples by either air current, silica gels, or incubation with cryoprotectant followed by rapid freezing or two-step freezing. It usually results in 100% recovery rate after liquid nitrogen drying in a laminar flow hood until 5–15% moisture content. For this technique, shoot tips or embryo are precultured on 0.3–0.6 M sucrose medium for 1–3 days. This is followed by encapsulation into alginate beads and treated with highly concentrated sucrose solution (approx. 0.8 M) for 16 h. These treatments induce tolerance in the samples. Following the pretreatment, plant genetic materials are dehydrated on silica gels or in a laminar flow cabinet to reach their optimal hydration levels. The advantage of this method is that it eliminates the need for other cryoprotectants that have been implicated in inducing genetic changes after cryopreservation such as DMSO and ethylene glycol [12].

5.3 Programmed freezing

With this method, samples are pretreated in cryoprotectants. The cryoprotectant agents used include DMSO, ethylene glycol and sucrose alone or in low concentration mixtures. Pretreated samples are dehydrated while frozen slowly (0.3–1°C/min) between −40°C and −70°C, then plunged directly into liquid nitrogen. This method

is based on the principle of free-induced dehydration. Programmed freezer that is expensive is required and it is a major disadvantage. Additionally, relatively long exposure of samples to subzero temperatures, which can be deleterious for cold-sensitive species is also a disadvantage [16].

5.4 Vitrification

The physical process by which a highly concentrated cryoprotective solution super cools to very low temperatures and finally solidifies into a metastable glass without undergoing crystallization at a practical rate. It was proposed as a method for the cryopreservation of biological materials because it avoid the potentially detrimental effects of extracellular and intracellular freezing [25]. This process involves pre-culturing of plant tissues on basal medium supplemented with cryo-protectants, pre-treatment with loading solution, dehydration with PVS, and rapid freezing rewarming. In general, vitrification protocols have been very useful for cryopreserving complex organs like shoot tips, and somatic embryos that could not be effectively frozen following classical protocols. The vitrification method uses a highly concentrated solution. This solution sufficiently dehydrates tissues and does not lead to injury. This leads to the formation of a stable glass along with the surrounding highly concentrated solution plunged in liquid nitrogen. Cells or shoot tips must be sufficiently dehydrated with highly concentrated vitrification solution at 0°C or 25°C and should not lead to injury. Recovery rate is 74.5% with 5-day pre-culture on 0.5 M sucrose followed by PVS2 treatment for 1 h at 0°C. This method has been applied to several plants that includes tropical and subtropical species. It has been applied in the cocoa industry through cocoa somatic embryos [12].

5.5 Droplet-vitrification

Droplet-vitrification is a protocol derived from combination of droplet procedure with droplet freezing technique. With regards to all the steps, droplet-vitrification is similar to vitrification method but the only difference is that materials are cryopreserved on foil strips in drops of vitrification solution. It has been successfully used for rubber shoot tips. It has a relatively lower recovery rate of 43% regrowth with pre-culture on basal + proline (0.193 M) for 24 h in the dark at 25°C and PVS2 15 minutes at 0°C.

5.6 V cryo-plate

This method involves the culturing of plant material such as nodal segments in the case of potato on solid MS medium containing 30 g/l sucrose and 0.3 g/l $CaCl_2$ at 20°C for 2 weeks. The shoot tips are then excised from the *in vitro* grown shoots and pre-cultured on MS medium containing sucrose at 25°C overnight. Pre-cultured shoot tips are then placed on aluminium cryo-plates with ten wells and embedded with calcium alginate gel. The next step is the performance of osmo-protection by immersing the cryo-plates for 30 minutes at 25°C in 25 ml pipetting reservoirs filled with MS medium with 2 M glycerol and 0.8 M sucrose. For dehydration step, the cryo-plates are transferred and immersed in another reservoirs filled with PVS2 for 30 minutes at 25°C. This is followed by the transfer of the cryo-plate in an uncapped 2 ml cryotube

in liquid nitrogen and immersed in a 2 ml cryotube and directly plunged into liquid nitrogen. The cryo-plate is then retrieved for rewarming in the in liquid nitrogen and immersed in a 2 ml cryotube containing 2 ml MS basal medium with 1 M sucrose, in which it is incubated for 15 minutes at room temperature. Rewarmed shoot tips are placed on solid MS medium and cultured under standard conditions [6].

6. Future trends and challenges

There have been several advancements in the identification and use of CPA in cryopreservation procedures. However, CPA toxicity remains a challenge in cryopreservation techniques. Mechanisms of the toxicity of CPA has not been understood fully [1]. Researchers are still working to better understand how different protective chemicals work to protect cells from the rigid temperature of liquid nitrogen.

Cryopreservation protocols have been developed for several crops. However, the number of crops represented in cryo-banks is still limited. Also the ability to successfully repeat the protocol in another laboratory has been a challenge. There is still more room for improvement for the cryopreservation of vegetatively propagated crops and the system requires a lot of optimisation. There is also the need for the development of efficient protocols, challenges related to cryo-banking capacities such as insufficient funding, lack of equipment and infrastructure, inadequate skilled personnel with knowledge on plant genetic resources [10]. The need for the acceleration of plant cryopreservation procedures especially for vegetatively propagated crops requires the development of global expertise. There should be a community of practice initiative involving curators of gene banks, researchers, advocacy organizations, academic institutions and other stakeholders to address the unmet need for cryopreservation advances. Other challenges should be outlined, underinvestment and untapped opportunities should also be identified [14]. There is the need for the establishment of pollen cryo-banks to facilitate a regular supply of pollen to support breeding programs for anther culture activities. The Biotechnology Laboratory at the Council for Scientific and Industrial Research (CSIR)-Crops Research Institute (CRI) in Kumasi, Ghana in sub-Sahara Africa receives germplasm of vegetatively propagated crops such as sweetpotato and cassava from research scientists in Ghana, CIP, International Institute of Tropical Agriculture (IITA) and other centers of the Consultative Group for International Agricultural Research (CGIAR). The current practice for preservation of these plant materials is conservation using slow growth media. Moving forward, the Biotechnology Laboratory in Ghana, should work on developing cryopreservation protocols for preservation of germplasm using liquid nitrogen and cryogenic refrigerators. Also, there is the need for the development of cryotherapy protocols for virus elimination of vegetatively propagated crops sent to the laboratory for *in vitro* propagation and long-term conservation.

A new threat for conserving global biodiversity in addition to other human activities that could lead to global mass extinction of germplasm is climate change. Recent approaches to *in situ* conservation are not very reliable to address anticipated changes. Therefore, there is the urgent need for the creation of new cryogenic models, protocols and technologies to mitigate the threats of climate change. Since cryopreservation is the safest and most cost-effective strategy for long-term conservation of germplasm of economically important plant species as well as endangered species [16].

7. Conclusions

Cryopreservation has been in existence since 2000 BC as demonstrated in archaeological findings that icehouse were used throughout Mesopotamia to store foods. In simple terms cryopreservation refers to the study of life at low temperatures. Plant cryopreservation on the other hand refers to conservation method for long-term storage of tissue samples at very low temperatures of −135°C to −196°C. The risk of causing variation is usually less. Cryopreservation methods are suitable are very useful for long-term storage of in vitro cultures of secondary metabolite cell cultures, embryonic cultures, clonal germplasm, endangered species, and transgenic products. The advantages of cryopreservation of plant genetic materials are enormous with several advantages of cryopreservation over other methods. Cryotherapy for virus elimination hold a lot of potential for crop germplasm dissemination.

The development of cryopreservation protocols are enormous. However, the number of crops represented in cryo-banks are still limited. For the acceleration of cryopreservation, there is the need for the development of global expertise. It is recommended that a community of practice initiative involving gene banks, researchers, advocacy organizations, academic institutions and other stakeholders come together to address the gaps in cryopreservation advances

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Conflict of interest

The authors declare no conflict of interest.

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Cryopreservation is the preservation of cells at sub-zero temperatures. Although useful, the process is not without its challenges. One of the major challenges is the formation of ice crystals in the cells to be preserved. However, vitrification, where glass is formed instead of crystals, can be achieved using cryoprotection agents (CPAs). This book provides a comprehensive overview of cryopreservation and its applications. It discusses advancements in the field, challenges, guidelines, and recommendations for successful germplasm conservation. Chapters discuss the use of CPAs, cryopreservation of fish sperm, cryopreservation of oocytes and sperms, female fertility preservation, cryopreservation of large structures and tissues, and much more.

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