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Cryopreservation Biotechnology in Biomedical and Biological Sciences

Edited by Yusuf Bozkurt

CRYOPRESERVATION BIOTECHNOLOGY IN BIOMEDICAL AND BIOLOGICAL SCIENCES

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Contributors

Sankha Bhattacharya, Miloš Bohoněk, Francisco Javier Henao, Julian Valencia, Zvjezdana Marković, Darko Preiner, Domagoj Stupić, Željko Andabaka, Iva Šikuten, Petra Štambuk, Jasminka Karoglan Kontić, Edi Maletić, Christiani Amorim, Ellen Leonel, Carolina Lucci, Mitchel Schiewe, Steven Mullen, Kenny Rodriguez-Macias Wallberg, Max Waterstone, Amandine Anastácio, Yusuf Bozkurt

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Meet the editor

Prof. Yusuf Bozkurt holds an Agronomy Engineering degree and MSc and Ph.D. degrees from Ankara University. He is currently working as Professor of Biotechnology of Reproduction in the field of Aquaculture at the İskenderun Technical University. His research interests are mainly related to reproductive biotechnology, particularly in cryopreservation of gametes in aquatic species.

Contents

Preface

Cryopreservation is the process of freezing and storing biological material at ultra-low temperatures (such as -196^{0}C) in liquid nitrogen (LN_2) for unlimited periods. Thanks to the application of this biotechnology, all biological activities including the biochemical reactions leading to cell death and DNA degradation discontinue. From this point of view, cryopreservation biotechnology focuses on preservation of cells that have many applications in the fields of human and veterinary medicine, agriculture and aquaculture. In addition, this form of biotechnology also has many applications in biomedical research, specifically in the areas of immunology, virology, neurobiology, toxicology and the pharmaceutical industry.

Cryopreservation Biotechnology in Biomedical and Biological Sciences describes principles and applications of cryopreservation biotechnology in different research areas and includes seven chapters that have been written by experts in their research fields. The book chapters are divided into four sections.

Section I, "Mechanism of Cryopreservation" contains one chapter entitled "Cryoprotectants and their usage in cryopreservation process", which describes the importance and mechanism of the cryopreservation process and also its comparison to vitrification. In addition, the functions and physical and chemical properties of cryoprotectants are discussed.

Section II, "Application of Cryopreservation in Human Medicine Researches" is divided into four chapters. The first chapter, "Clinical outcomes of assisted reproductive techniques using cryopreserved gametes and embryos in human medicine", provides valuable information regarding fertility treatments in human reproductive medicine, focusing on cryopreservation of spermatozoa, oocytes and embryos. The second chapter, "Cryopreservation of platelets: advances and current practices" focuses on the long-term preservation of platelets, which is necessary for the coagulation of blood and stopping bleeding. The third chapter, "Cryopreservation of preantral follicles", provides valuable information regarding the structure and development of ovarian follicles. Beside this, advantages and necessities of using oocytes in preantral follicles for the aim of cryopreservation are explained in humans and mammals. The fourth chapter, "Vitrification: Fundamental principles and its application for cryopreservation of human reproductive cells", is related to mechanisms of cryopreservation in terms of application of this technique in human medicine with emphasis on reproductive cells. In this way, mechanisms underlying the problem of the intra- and extracellular ice formation, the role of cryoprotectants, stages of the warming and cooling process and use of this biotechnology in the field of IVF industry are explained.

Section III, "Application of Cryopreservation in Veterinary Medicine Research", contains one chapter entitled "Biological signals of sperm membrane resistance to cryoinjury in boars",

discusses biological mechanisms before the cryopreservation/vitrification process causing cryoinjuries in sperm cells in boars.

Section IV, "Application of Cryopreservation in Agricultural Research", contains one chapter entitled "Cryopreservation protocols for grapevine shoot tips", which presents valuable information regarding cryopreservation of shoot tips of grapevines, which is an economically important agricultural product.

This book covers different applications of cryopreservation biotechnology. I hope that this book will be helpful for researchers studying cryobiology and related issues. I would like to thank all the authors for their distinguished contributions, IntechOpen Publishing Company, and its Author Service Manager Ms. Dolores Kuzelj for her help in publishing this book.

Prof. Yusuf Bozkurt
İskenderun Technical University
Faculty of Marine Sciences and Technology
İskenderun, Hatay, Turkey

Chapter 1

Introductory Chapter: Application Fields of Cryopreservation Biotechnology

Yusuf Bozkurt

Additional information is available at the end of the chapter

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1. Cryopreservation biotechnology

Cryopreservation is the process of freezing the biological material at a temperature of liquid nitrogen (LN_2) (−196°C). This means biological activities discontinue including the biochemical reactions creating cell death and DNA damage at these low temperatures. In this way, it is possible to store the biological materials unchanged for centuries with the capability of recovering of the cell functionality following the thawing process.

The cells that were chosen for the early studies on the effects of freezing and thawing on cell viability were gametes. First cell material was "sperm cells" because of their availability, small size, and motility that was a simple marker of viability. Second cell type was "oocytes" due to their size large enough to allow for simple morphological evaluation.

Spallanzani [1] for the first time reported successful sperm freeze-thaw application on stallion semen. Nowadays, there are numerous cryopreservation protocols varying in terms of extenders, storage temperatures, freezing/thawing periods, and biological samples, which are suitable for the cryopreservation process.

The success in cryopreservation of biological materials has been gradually increasing every year with the understanding of physical and chemical process occurring during the freezing and thawing cycle. From this point of view, it is well known that intracellular ice formation, especially, is an important issue that has to be controlled to keep the cell membrane undisturbed and the cells lively. The critical issues for prevention of ice formation are the freezing rate and freezing medium composition. The freezing medium is known as a cryoprotectant containing extender solution. The choice of cryoprotectant and its concentration show differences between cells and species and influence the cryoprotection results.

Finally, cryopreservation biotechnology focuses on preservation of cells that have many applications in the fields of human and veterinary medicine, agriculture, and aquaculture. In addition, this biotechnology has many applications in biomedical researches, specifically in the areas of immunology, virology, neurobiology, toxicology, and pharmaceutical industry.

2. Application of cryopreservation biotechnology in medical sciences

In human medicine, cryopreservation has gained its importance when its usage in infertility treatment was realized. Since then, gamete cryopreservation has been developed to solve fertility problems in this field.

Sperm was the first type of reproductive cell successfully frozen and still remains the easiest cell to freeze because of containing low amounts of cytoplasm and consequently low quantity of water. Furthermore, sperm nuclear material is compressed and preserved against physical injuries. For these reasons, cryopreservation of sperm cells gives excellent results in terms of viability and fertility and is widely used in human medicine today.

In recent years, protocols regarding freezing of semen and embryos were established successfully and live births from assisted reproductive cycles using frozen semen or embryos were recorded. In addition, researches have also focused on the cryopreservation of human oocytes and ovarian tissues. While there are still insufficient researches especially for the oocytes, studies on the cryopreservation of immunological memory lymphoid cells, aortic root allografts, and osteoblasts for bone banking are going on. Cryopreservation of cornea, umbilical cord, and hematopoietic cells and sperm banking procedures are performed routinely in the field of human medicine [2].

In veterinary medicine, preservation of gametes is closely connected with the development of artificial insemination. Today, reproductive biotechnologies such as artificial insemination used in breeding programs are well developed.

During the last 100 years, many hundreds of species have been lost and a third of the breeding animals are threatened with extinction. Concerning with the threatened species, cryopreservation of genetic material (sperm, egg, and embryo) is used for the genetic management programs and genetic resource banking [3].

Since the first successful cryopreservation of bull semen [4], it is being used to reproduce scarce and threatened species. Numerous bovine calves have been produced via transferring of the cryopreserved embryos into cow, which are artificially fertilized with the frozen-thawed bull sperm greater than 25 million each year [5]. Nowadays, tissues, cultured cell lines, DNA, and serum samples could be frozen and stored in cryogenic banks.

3. Application of cryopreservation biotechnology in biological sciences

Cryopreservation is one of the most reliable methods for long-term conservation of plant genetic resources, because all metabolic processes and physicochemical changes are suspended at the cryogenic temperature (−196°C).

This biotechnology mostly interested with the germplasm cryopreservation is used for the genetic improvement of domestic varieties and their well adaptation to environmental changes in the field of agriculture. In spite of preservation of plant germplasm in cryogenic conditions is comparatively a new practice, a range of cryopreservation techniques for the conservation of plant cells and tissues were developed by the scientists more than 40 years [6]. Nowadays, it is a feasible application of using these techniques for the plant genotypes. A large number of laboratories are constituting this biotechnology for the aim of preservation of genetic resources. Recently, new cryogenic procedures using cryoplates (the V cryoplate and D cryoplate) have been developed. These methods provide some advantages such as ease of handling during the application and high regrowth rates, following cryopreservation process.

In addition, gamete, embryo, and embryonic cell cryopreservation has become a tremendous value in aquatic biotechnologies, which provide an important tool for the propagation of economically important species and also in protection of the endangered species and genetic diversity in aquatic species.

Since the first work of Blaxter [7] with Atlantic herring spermatozoa, sperm cryopreservation protocols are now available for over 200 finfish and shellfish species.

According to results of the researches, cryopreservation of sperm from marine fish species is more successful when compared to those obtained from the freshwater fish, and fertilization rates are similar to those obtained with mammalian species [8].

4. Conclusion

Cryopreservation has many biotechnological applications in different fields. This situation has been increasing the importance of cryobiology as a science, examining the effect of ultralow temperatures on cell, tissue, organ, and organisms and also the freezability of these structures maintaining their viability [9]. It is possible to underline that better understanding of functional properties of thawed cells following freezing process has been accelerating development of the cryopreservation biotechnology.

Author details

Yusuf Bozkurt

Address all correspondence to: yfbozkurt@hotmail.com

Faculty of Marine Sciences and Technology, İskenderun Technical University, İskenderun, Hatay, Turkey

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Section 1

Mechanism of Cryopreservation

Chapter 2

Cryoprotectants and Their Usage in Cryopreservation Process

Sankha Bhattacharya

Additional information is available at the end of the chapter

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Abstract

Cryoprotectants are basically some chemical compounds which prevent cells or tissues from damage due to freezing. Mostly vitrification and thawing process are mostly used in cryopreservation. Now a day's cryopreservation is becoming an apex technology for Health sector, as many organ and tissue transplant methods has been developed in recent time. Organs or tissues need to preserve properly before implantation, hence cryoprotectants plays a key role modern cryonics. This article seeks to provide answers for how cryoprotectants works and prevent tissue damage from shrinking ice crystals during freeze drying or vitrification. The various animal tissues, organs, lymphocytes, cartilages, bone marrow, proteins can be stored by using proper cryoprotectants. Optimization of cryoprotectants is a challenging task, as elevated concentration may cause cytotoxicity. This article also highlights the implication, challenges and recent advances of cryoprotectants along with its types. The importance of cryobank system and its importance were also being emphasized in this article. Further, importance of cryoprotectants in nano suspension preparation was also been discussed in this article.

Keywords: cryopreservation, vitrification, cryoprotectants, cryobank system, dimethyl sulfoxide, glycerol

1. Introduction

To prevent cell death during preservation, tissues and organs temperature used to lower to stop cell death. But at the same time rate of cooling also affect the cell survival. The preserving cells which are cools quickly are susceptible for cell death due to rapid intracellular ice formation. Sometimes due to dehydration, cells can die. In other way, due to the presence of hypotonic solutions surrounding of cells can also leads to cell shrinking and cell death.

The deleterious affect associated with slow drying was sorted out by using glycerol as cryoprotectant during 1949. Without proper cryoprotectant cells ruptures due to "Solution effect" injury; in which residual solution of cells altars shape, mechanical strength of the cells followed by osmatic oscillation during freeze drying. What basically cryoprotectants do is after dissolving in preserving solution it lowers the melting point of the solution so that tissue or organs can freeze within its surroundings but not within its cells [1]. These cryoprotectants are sometime called as antifreeze. There are so many cryoprotectants but glycerol, propylene glycol, dimethyl sulfoxide (DMSO) are most commonly used within this category. For the cell survival after freezing and thawing of liquid nitrogen temperature, it became necessary to preserve tissues with suitable cryoprotectant (5–15%) which helps to form large unfreeze pockets [2]. These pockets helps to prevent crystal formation and mechanical damage. During freeze drying process organs and tissues are venerable to freezing injury, for any organ to re-established function after freeze drying has to have intact parenchymal cell to cell of small blood vessels. Only 25% of cell survival during freeze drying is not enough, hence a new approach of tissue preservation called vitrification is required to protect tissues. Vitrification means turns into a glass. Fathy, advocated vitrification, in which tissue or organ encircled into unfrozen glassy pocket containing cryoprotectant, it was planned, tissues to be treated with maximum cryoprotectant before freeze drying to avoid ice formation [3]. Due to the rapid acceptance of verification and slow freezing process, a maximum number of animal germ plasmas, tissues were started to store by these process. Further advancement embarks the idea to preserve tissues below −130 to −196°C, which is basically a sub-zero temperature using some substances. It was also observed that all the associated problems of refrigerating storage such as integrity of living cells while storing, freezing injury, forming ice crystals which eventually damage stored cells, could be restricted from using such substance. These substances are also helping in moisturizing the living cells by penetrating inside of stored cells without altering or creating any lethal effect to those cells, such substances are called as the cryoprotectants or the cryopreservatives. Without cryoprotectants at low-temperature biological cells damaged, and at in transition phase such as changes from frozen solid to a liquid by a general warming, cells loses numbness and stiffness, this condition is called thawing. Mostly slow freezing is preferable for preserving cells in which cells are frizzed slowly below its freezing point, but at a certain point, the formation of spherical crystals can destroy cells chronobiology. These crystals are called as unfrozen fractions [5]. Using cryoprotectants along with some electrolytes and sugars it is possible to increase osmatic strength of the solvent by which positive efflux of moisture towards the cells occur and during this process chances of intercellular ice, the formation becomes negligible. Farther cooling causes increase of viscosity and due to this, chances of formations of ice crystals would become very infinitesimal. In the last stage of slow cooling, remaining unfrozen fraction transformed to amorphous solids without ice crystals. The main objective of cryopreservation is to create a dehydrating environment for preserving cells. In this process cytosol observed less injury during cold freezing within liquid nitrogen, as numbers of crystalline substances formation would be very minimal. Mostly it was found that cryoinjuries occur during anomaly of instantaneous super freezing of cells. Slow freezing's with lucid cryoprotectants causes fewer injuries [6]. Eventually, cryoinjuries occurs during pre-freezing and post-thawing, within the temperature of 0–40°C. During cryopreservation technique, pH, osmotic pressure, and concentration

of cryoprotectant has to be maintained, as elevation can cause cytoinjuries. In some cases, if the cell cytosol contains specific fatty acids, i.e., spermatozoa plasma membrane, it helps to maintain proper osmotic pressure and resist shock during thawing and freezing, by which fatty acids helps to maintain cells rehydration.

2. Toxicity due to cryoprotectant

During cryopreservation process using vitrification or freezing, almost half of the cell fluids were replaced with cryoprotectant molecules. These cryoprotectant molecules some time cause toxicity while in warm temperature. For example as a cryoprotectant in warm condition, propylene glycol is nontoxic while ethylene glycol metabolized to toxic elements. Recently it was found that lipophilicity of cryoprotectant could help in deeper penetration of cryoprotectant into cells causes destabilization of cells. On the other hand, strong hydrogen bonding also correlates with toxicity by disrupting the hydration shell around macromolecules. The electrical properties of cryoprotectant solution also has membrane toxicity. Among all cryoprotectants, DMSO is severely toxic, basically DMSO/formamide solution has been useful in vitrification process, but mechanism of biomedical toxicity reduction is still unknown [1, 4].

3. Various components of cryoprotectant solution

Cryoprotectants solution may be a freezing solution or sometimes it may be a carrier solution. The carrier solution should not be an explicit cryoprotectant. Carrier solution helps to provide minimum support for cells at freezing temperature. Carrier solution often comprise of nutritional salts, buffers, osmogens, and apoptosis inhibitors. Often ingredients maintains isotonic concentration (300 milliosmoles) so that cells neither swells nor shrink when held in carrier solutions. These carrier solutions sometimes called as "base perfusate" solution. Carrier solution consisting of M22 cryoprotectants is called as LM5. During the process of freezing the carrier solution concentration always remains constant. Like carrier cryoprotectants there is another type of cryoprotectants called penetrating cryoprotectants, the basic role of penetrating cryoprotectants is to reduce cell dehydration and ice growth. The penetrating cryoprotectants are the major ingredients of vitrification solutions. Certain large molecular polymers are also been added to cryoprotectant solutions, they literally inhibits ice growth using same mechanism of penetrating cryoprotectants, these are called as Non-penetrating cryoprotectants. Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) are examples of non-penetrating cryoprotectants. These cryoprotectants are highly toxic as compare to penetrating cryoprotectants in same concentration. Recently it was discovered that non-penetrating cryoprotectants can increase tonicity of vitrification solutions, which helps to prevent chilling injury.

Some ingredients in cryoprotectants solution called ice blockers, which are helping directly to block ice growth. Some examples are polyglycerol, polyvinyl alcohol, X-1000 and Z-1000. Ice blockers are used only in vitrification solution [4, 6–8].

3.1. Utilization of cryoprotectants

During cryopreservation, initially nearly 10% cryoprotectants were added in a single step. This causes classic shrink swell response of cryobiology, in which cells first shrink due to osmosis and later swell by penetrating cryoprotectants enters the cell. Unless and until cryoprotectants volumes equals between intra cellular and extra cellular fluids of cells swelling persist. Within 10 min of concentration and volume adjustment, cell or tissue is ready for freezing. The cooling process is done very slowly, mostly less than 1°C/min. The addition of vitrification solutions during cryopreservation should not be exist 50% in a single steps, because of elevated osmatic shrinking response. The material's which need to be vitrified exposed to several cryoprotectant solutions such as 1/8×, 1/4×, 1/2×, 1× full concentration vitrification solution as specially for 20 min each steps. During vitrification cooling and rewarming are done rapidly. During this process cryoprotectant solutions just perfused through cells like blood flow in blood vessels. After vitrification, cryoprotectant can be remove by reversing the steps describe earlier [9–13].

4. Mechanism of cryopreservation

The melting point of water decreases after proper mixing with suitable cryoprotectant. The various anti-freezing cryoprotectants are dimethyl sulfoxide (DMSO), propylene glycol. During freezing and thawing of isolated cells mostly 5–15% concentration of cryoprotectant concentration has to be maintained for better result. With cryoprotectant the freezing of cells in liquid nitrogen would be slower, further formation of relatively large vesicles and fewer salt crystals helps to protect cells from any mechanical injuries. As per evidence cryoprotectants usually do not forms any hydrates and they are relatively less toxic to cells in elevated concentration. Vast expensive use of glycerol as cryoprotectants becomes a stepping stone towards finding more than hundred more cryoprotectants within 50 years. In new age penetrating cryoprotectants were using vastly in the pharmaceutical industry because they are having a molecular mass of fewer than 100 Da and they penetrate inside of the cells and maintain moisture during freeze drying [4, 9].

5. Some common cryoprotectants used in pharmaceutical preservation

5.1. Dimethyl sulfoxide (DMSO)

The Great Russian scientist Alexander Zaytsevin on 1866 synthesized DMSO [4]. Basically DMSO is an organosulfur compound with the formula $(CH_3)_2SO$. It is also a polar aprotic solvent which can dissolve polar and nonpolar compounds and can be easily miscible with wide range of organic solvents and with water. DMSO has low cost and minor cytotoxicity, which makes it more prominent candidate for cryopreservation. It has garlic like taste. At any particular temperature, DMSO reduces the electrolytic concentration in the residual chilled

contents in and around of a biological cell, during cryopreservation. However rising of altered demarcated cells due to DNA methylation and histone alteration is a drawback of DMSO based cryopreservation. DMSO has typical property; it freezes within 18.5°C. This means, below room temperature DMSO transformed into solids, and this property makes it most suitable for cryoprotectant.

5.2. Ethylene glycol

Voelkel et al. [27] used 1.5 M ethylene glycol as a cryoprotectant for bovine embryols allowing direct transfer of frozen-thawed embryos to recipient females. Ethylene glycol alters the hydrogen bonding when it mix with water. Purified ethylene glycol has a freezing point at about −12°C, but after mixing with 40% water and 60% ethylene glycol the freezing point of the mixture would depressed and mixture becomes incapable of forming crystalline substances. This condition leads to transformed freezing point at −45°C. This property of ethylene glycol makes it the most effective candidate for cryoprotection. But some toxicity was observed for ethylene glycol, like GI irritation, pulmonary edema, and lung inflammation.

5.3. Glycerol

Oana Lelia Pop et al. [28] studied the effect of glycerol as cryoprotectant during freeze drying of microspheres containing probiotic cells. The colorless, the odorless viscous liquid of simple polyol (sugar alcohol) compound named as glycerol or glycerine. Glycerol has good kosmotropic properties; it forms hydrogen bonds with water molecules. This condition makes difficult to form ice crystals by mixture (70% glycerol and 30% water), unless and until the temperature is very low such as −37.8°C. Compare to other cryoprotectant glycerol is less toxic in high concentration.

5.4. Propylene glycol

Propylene glycol IUPAC name is propane-1, 2-diol. It is basically a non-irritating synthetic organic compound with a chemical formula $C_3H_8O_2$. It possess a fairly sweet taste with color less and odorless characteristics. Chemically it is a diol and miscible with water, chloroform, acetone. It is widely used as aircraft deicing fluid. Propylene glycol is sold under the name of RV or marine antifreeze. It also carries the property of automotive antifreeze.

5.5. 2-methyl-2, 4-pentanediol (MPD)

MPD widely used as precipitant, but in protein crystallography, it can be used as a cryoprotectant. It can be vividly used with polar and non-polar solvent. It can help the protein to precipitate.

5.6. Trehalose

This compound comprising with two molecules of glucose. Trehalose is otherwise called as mycose or tremalose. Due to its high water retaining properties it can be used as cryoprotectant.

Trehalose is less soluble than sucrose, except at high temperatures (>80°C). Trehalose forms rhomboid crystals of 90% calorific sucrose. The anhydrous forms of trehalose readily regain moisture to form the dehydrate. Trehalose improves cell survival after thawing compared with the standard freezing procedure. Sometimes trehalose can be used along with hyaluronic acid to treat dry eye.

5.7. The cell bank series

The cell banker series containing cryoprotectants media has 10% DMSO, a specific polymer, pH modifiers, glucose, bovine serum albumin. This combined cryopreservatives are used to preserve mammalian cells. This techniques allows rapid cell cryopreservation at −80°C and mean survival rate of cells after freezing and thawing was outstanding.

5.8. Formamide

It is also known as methanamide. It is derived from formic acid. It has ammonia like odder and clearly miscible with water. As a cryoprotectant, it helps to soften tissue vessels. It is also been used as a resins and plasticizer. The most common formamide is called as dimethylformamide, $(CH_3)_2NCHO$.

5.9. Glycerol 3-phosphate

This chemical compound comprising of glycerophospholipids. This product is also called as 3-O-phosphonoglycerol.

5.10. Proline

Proline catalyzes aldol condensation. L-Proline is an osmoprotectant and therefore it has versatile use in Pharmaceutical and Biotechnological applications. Proline is actively participating in biosynthesis of protein. Proline has α-amino group, a α-carboxylic group, pyrrolidine side chain, aliphatic amino acid group.

5.11. Sorbitol

Sorbitol is otherwise called as glucitol. The best part of sorbitol is, its get metabolize in tissue very slowly. During reduction of glucose aldehyde group reduced to hydroxyl group. Mostly sorbitol is prepared from corn syrup. Basically sorbitol is an isomer of mannitol. Apart from good cryoprotectant property sorbitol also used for hyperkalemia.

5.12. Diethyl glycol

It is an organic compound with a formula $(HOCH_2CH_2)_2O$. It is colorless and odorless poisonous hygroscopic compound. It is miscible with water, alcohol, acetone and ethylene glycol. Diethyl glycol is produce by partial hydrolysis of ethylene oxide. The dilute solution of diethyl glycol is a good cryoprotectant. A dilute solution of diethylene glycol can also be used

as a cryoprotectant; however, ethylene glycol is much more commonly used. Most ethylene glycol antifreeze contains a few percent diethylene glycol, present as a byproduct of ethylene glycol production.

5.13. Sucrose

Sucrose is actually naturally occurring carbohydrates sucrose in low temperature (−45°C), provides required nutrition to preserved cells, sucrose with the combination of DMSO maintains good cyroprotection properties.

5.14. Triethylene glycol

It is a colorless odorless viscous liquid with a molecular formula as $HOCH_2CH_2OCH_2CH_2OCH_2CH_2O$.

5.15. Polymers

It is possible to trauma biological materials using selective cryoprotective agents. Among all encapsulating materials, non-diffusible synthetic polymers can provide ridge cryoprotections of the biological cells within. For example polyvinyl alcohol, PEG, hydroxyethyl starch has good tendency to decrease the size of ice crystals.

6. Type of cryopreservation

6.1. Isochoric cryopreservation

Mostly all cryopreservation is based on an isobaric (constant pressure) process where freezing occurs at 1 atm pressure. But it has its own disadvantages, in this process intercellular ionic concentration increase, due to which chemical integrity changes within the cells during freezing, which causes cell damage. But in the isochoric (constant volume) process the metabolic rate of frizzing constantly changing with every 10 degrees of temperature reduction. In the isochoric process, it is possible to store at absolute zero temperature and it helps to maintain cell integrity during slow frizzing [14].

6.2. Isobaric cryopreservation

Isobaric cryopreservation technique deals with the preservation of cells in one atmospheric pressure. This process is widely used, but the certain limitation of cyto cellular damage makes it more bizarre.

6.3. Hyperbaric cryopreservation

This cryopreservation method maintains low temperature (0°C) and high pressure followed by gradual demotion of pressure which causes rapid freezing. Hyperbaric preservation

allows cells to be maintained at a low temperature without freezing and in an aqueous phase with one study reporting that survival was a function of both compression rate and final pressure magnitude. Despite the deleterious effect of pressure maintenance, some tissues and cells shown good preservation. For example red blood cells survived pressure up to 200 MPa, where else liver up to 35 MPa. By increasing elevated pressure it reduces temperature to sub-zero Celsius [15–17]. This condition leads to rapid freezing of biological tissues, by which many tissues can be stored by this process, i.e., kidney (10,000 atm), cells (200 atm), and liver (70 atm).

7. Optimizing freezing

Freezing or cooling optimization is a big issue in cryopreservation. Optimizing freezing condition with cryoprotectant needs 3–4 h interventions. The slow crystal formation process called seeding can damage cellular integrity. Optimizing freezing rate and concentration of cryoprotectant are very important for long storage of cells.

If cells were cooled very slowly and steadily, then extracellular fluids of the cells form ice crystals which pinch the cells to withdraw all intracellular component.

At elevated −350°C temperature cells can lost its structure and shape. On the other hand, rapid cooling can cause serious damage to intracellular integrate of cells, where cytosol, parenchyma, nucleus and almost all cellular component rapidly freeze, which causes intracellular formation. All the integral parts of cells may come out and cell may die due to shrinking [18–20].

Hence, it is important to optimize cryoprotectant concentration with cooling rate. The optimize cooling requires the optimum intake of cryoprotectant within the cells and maintaining the integrity of the cells by not forming ice crystals while freezing, for example, human oocytes and embryos need minimum 90 min for preservation. Certain limitation of slow cooling makes it more challenging, such as it takes more sophisticated instruments with more time for cooling and at the same time certain tissues such as in vitro derived bovine, pig embryos, human MII oocytes, and blastocysts are very susceptible to chilling injuries.

7.1. Carrier solution or base perfusate used in cryoprotection

The carrier solutions acts as a life support or buffering substance for preserving cell. The main purpose of carrier cell is to maintain life integrity in cells during near freezing condition. Carrier solutions constituted with some essential buffer, osmogens, nutritional elements, salts and certain apoptosis inhibitors. To maintain stability within the cells, carrier solution maintains iso-osmolarity, which makes cells not to lose its original shape. For example M22, cryoprotectant solution is used with specific carrier solution is called as LM5. Most importantly carrier solution concentration cannot be altered with altering cryoprotectant concentration. It should remain constant, based upon the cells to be preserve.

7.1.1. Ice blockers

Ice blockers are the substance which prevents ice to grow. While temperature is low and pressure is high this ice blocker binds with contaminants or ice inculcator to cleave ice formation. Examples are low molecular weight polyvinyl alcohol, polyglycerol, called as X-1000 and Z-1000. Mostly ice blocker is used in vitrification process.

7.2. Cryopreservation using nanosuspension

Nae-Oh Chung et al. [29], had tried to give an in-depth understanding of aggregation process of nanoparticles freeze-drying technique, they also outlines the importance of Cryoprotectants during lyophilization process. Yancai Wang et al. [30] had tried to give an outline of choosing cryoprotectant in freeze dried nanosuspension with suitable functional stabilizers. Cryopreservation and freeze drying both are very efficient ways to improve long term stability of nanosuspension. In Yancai Wang et al. work, mannitol (5% w/v), trehalose (5% w/w), lactose (7% w/v), sucrose (10% w/v) was considered as an optimized cryoprotectants for preparing resveratrol (RSV) and quercetin (QUE) nano suspension. During this process of preparing Nano suspension using anti-solvent precipitation method, D-α-tocopheryl polyethylene glycol succinate (TPGS) and folate modified distearoylphosphatidyl ethanolamine-polyethylene glycol (DSPE-PEG-FA) was used as a functional stabilizers. Upon storage for 90 days, it was observed that RSV & QUE nano suspension containing mannitol 5% w/v has limited deviation in particle size, enhanced dissolution rate, good physical stability as compare with other optimized cryoprotectants containing RSV & QUE nano suspensions.

8. Recent advances in cryopreservation

Due to recent advancement of organ and tissue transplantation, it is important to have cryoprotectants research. Roger Gosden [31] did some extensive research on preserving ovarian tissues. He used 0.1 M fructose and dimethyl sulfoxide (DMSO) to preserve seven rat ovaries. It takes over 30 min to freeze and stored in liquid nitrogen overnight before thawing. Arav et al. [19] develops a novel technique using 1.4 M DMSO (10% by volume) for preserving ovaries. This device transplanted in a test tube where freezing of organs can be possible between 0 and −35°C. This device far end is super cooled and stable. The organs which is about to preserve at first must be freeze in liquid nitrogen than exposed and thawed to 68°C for 20 s than 37°C for 2 min. In this process preserved organ shown immediate blood perfusion after transplantation. Dittrich et al. [20] developed a method by freezing pig uteri in near dry ice temperature. It was tried to prove that this process is most efficient then vitrification technique. Viatcheslav Berejnov et al. [21] studied the effect of cooling rate on vitrification of aqueous solution. It was observed that T = 295 K to T = 77 K temperature, vitrification is reported in liquid nitrogen for almost all the cryopreservatives. It was concluded and confirmed that by X-ray crystallography, the transition from polycrystalline to vitreous occurs with in the span of 2% w/v in cryopreservatives. By which polycrystalline ice from hexagonal to cubic depends upon the elevation of cryopreservatives cooling rate and concentration. Anderson et al. [22]

studied about ovarian cryopreservation for fertility preservation. They concluded that oocyte cryopreservation technique has more advantages as it produces approximately 100 fold more babies as it can preserve large number of oocytes within primordial follicles without further hormonal therapy. Chong et al. [23] studied about cryopreservation of neurospheres which is derived from human glioblastoma multiforme. Cryopreservation of neurospheres cried out by using 90% serum and 10% dimethyl sulfoxide. Further vitrification yields self-renewal and multi potential properties. Xu et al. (2010) studied about the role of apoptotic pathway in the low recovery rate after cryopreservation of dissociated human embryonic stem cells. It was observed that recovery of cells after cryopreservation of human embryonic stem cells (hES) become a huge challenge. It was found that the generation of reactive oxygen species (ROS) is significantly increased with F-actin altered distribution. Further analysis shown the activation of and caspase-8 and caspase-9, which causes increase in cellular toxicity. Cetinkaya and Arat [24] studied about bio-banking system of cryopreservation for cartilage and tissue cells. Vitrification is used to preserve primary adult cartilage and fatal cartilage cells. The cartilage cells were slowly freeze using ethylene glycol (EG), Ficoll and sucrose. During slow cooling three different cooling rate were set as 0.5, 1 and 2°C/min. Results astonished all by no significant changes in viability rations, proliferative activity and GAG synthesis observed after vitrification (1°C/min). This study emphasis the needs of vitrification in bio-banking of cell donor sources in nuclear transfer studies. Wong et al. [25] did vital studies on human embryos cryopreservation and its fertilization rate. In modern days cyroprotection human embryos cells is routine work, but optimizing success rate of transplantation is a matter of question. In this study high quality randomized trials were used to find best cryoprotectant protocol for fresh transfer of embryos. Silva et al. [26] reviewed about mammalian conservation biology and cryopreservation. They enlightened the importance of germ plasma cryopreservation of embryos, gonadal tissues, gametes, and some somatic tissues. They also dignified the importance of more usage of cryobanking for the preservation of cells and tissues of threatened species.

8.1. Application of cryopreservation

Now a days cryoprotectants has some versatile use. Mostly in preserving organ cells, in molecular biology, cryosurgery, blood transfusion, artificial insemination, bone marrow transplantation, *in-vitro* fertilization and most recently in identifying unknown transmissible disease or pathogen. During 1996, first embryo preservation for fertility was took place using IVF cycle. IVF cycle was prerequisite before chemotherapy of breast cancer diagnosed patients. Preserving oocytes using cryoprotectants is already an established technique. This technique causes no potential side effects on parturition. Same way, sperm and semen can be used after proper cryopreservation. Preserving testicular gonads using cryopreservation is still in infancy, but in future cryopreservation can be used in vasectomy.

8.2. Limitation of cryopreservation

In cryopreservation technique, at −196°C in liquid nitrogen cells stops metabolizing which leads to unavoidable side effects. Sometimes there will be slow genetic changes occurs within

the biological cells associated with the lipids and proteins, that could disfigure integrity of the cells. Often it was witnessed that, cryoprotective agents could damage chromosome stability of cells, like in higher concentration DMSO could produce tumor and also some times cryoprotectants makes cells susceptible towards infections.

9. Conclusion

Cryobiology will have a significant impotence in near future as a number of transplantation of vital organs of humans are becoming more demanding in the modern age. Yet, so many important complications are to be mitigated, like the exact mechanism of cryoprotectant, mode of action and cellular interactions with potential toxicity. More futuristic research is needed in respect of optimizing cryoprotectant concentration and slow and high freezing process. It was observed that macromolecular cells can easily preserve by cryoprotectant but as far as large tissues or organs (kidney, liver, heart, lungs, and skin tissues) are concerned, the success rate of freezing is not up to the mark by using cryoprotectant. Further optimized research on selection of specific concentration of cryoprotectants, freezing and thawing rates, best infusibility, and equilibrium times in cryopreservation might lead to better cell survival during preservation. Modern emerging cryobank is a promising approach towards organ transplantation. But still, standardization and optimization techniques are warrant for best cryopreservation.

Author details

Sankha Bhattacharya

Address all correspondence to: sankhabhatt@gmail.com

Department of Pharmaceutics, ROFEL, Shri. G. M. Bilakhia College of Pharmacy, Gujarat, India

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Section 2

Application of Cryopreservation in Human Medicine Researches

Chapter 3

Clinical Outcomes of Assisted Reproductive Techniques Using Cryopreserved Gametes and Embryos in Human Medicine

Max Waterstone, Amandine Anastácio and
Kenny A. Rodriguez-Wallberg

Additional information is available at the end of the chapter

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Abstract

The methods of cryopreservation play a key role in assisted reproductive technique (ART) treatments, as they increase the efficacy of the treatments by allowing banking of supernumerary embryos for later use. It has been recently proposed that these methods could also increase the safety of ART treatments, by reducing complications such as ovarian hyperstimulation during early pregnancy; thus, the policy of total freeze for later differed transfer of embryos has been proposed. Also of great importance, cryopreservation of oocytes and spermatozoa has permitted gamete storage for long term facilitating practical routines such as the gamete banking for third-party reproductive treatment. In this chapter, the clinical indications and treatment outcomes will be revised and data updated on the safety of using cryopreservation methods in ART treatments.

Keywords: cryopreservation methods, assisted reproductive technique (ART) treatment, pregnancy, embryos, ovarian hyperstimulation

1. Introduction

The development of cryopreservation techniques has made possible the use of frozen and thawed gametes and embryos aiming at reproduction, by means of assisted reproductive techniques (ART). Cryopreservation allows to banking gametes for later use, including also the possibility to be used by other individuals, as in donor treatments. Effective techniques such as in vitro fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI), worldwide

applied, offer a high efficacy by the creation of supernumerary embryos. As recognised downsides of IVF/ICSI treatments include the high prevalence of perinatal complications due to multiple births, the recommended practice of transferring fewer embryos in the fresh IVF treatment cycle, with the goal of performing single embryo transfer and the cryopreservation of remaining embryos for their later use in frozen-thawed cycles, one at a time, is currently the trend [1]. The cumulative chance to achieve pregnancy and live-birth through IVF/ICSI treatments is thus enhanced by the later use of thawed embryos in separate treatments.

The methods for cryopreservation of embryos and gametes have demonstrated effective and safe, and have developed towards the achievement of a clinically established level. These methods are also currently being offered to patients suffering of cancer, due to the risk of infertility associated with certain cancer treatments, or to individuals with conditions that have an inherent risk of premature gonadal insufficiency and infertility, aiming at fertility preservation [2].

Although embryo cryopreservation has historically been regarded as the first-choice technique for fertility preservation, social, ethical and legal reasons usually restrict its use to couples who have entered into a committed long-term relationship. However, women without a partner may attempt this possibility using a sperm donor. Furthermore, this issue has also been shown to translate to fertility preservation undertaken electively, with one study finding that >80% of patients undergoing oocyte preservation by choice were single at the time, and that lack of a partner was by far the most common reason for not pursuing child-bearing earlier [3].

As such, the cryopreservation of gametes affords individuals an increased level of reproductive autonomy, and ensures that fewer patients are faced with the extraordinarily difficult decision of later reproducing with a partner who may no longer be ideal, or not reproducing at all. Here, the most common indications for gamete cryopreservation in both males and females and embryo cryopreservation will be discussed, along with their clinical outcomes, necessary considerations and future perspectives.

2. Current status of fertility preservation by cryopreservation of gametes

Although semen cryopreservation has remained an established technique for many years, the cryopreservation of mature oocytes was considered experimental by the American Society for Reproductive Medicine (ASRM) until 2013 [4]. As such, this relatively recent development has paved the way for an explosion of social fertility preservation ('social freezing'), found by a HFEA report to have increased more than two-fold between 2013 and 2016. In fact, a 10% increase in the number of egg freezing cycles was reported from 2015 to 2016 [5]. In a field which was once dominated by fertility preservation following medical diagnosis, this represents a dramatic paradigm shift, which must be regulated to ensure transparency for, and protection of, the prospective patient. This differs from traditional approaches to non-elective fertility preservation, where patients who may be younger or have no desire to delay child-bearing are faced with a high likelihood of complete infertility following resolution of their disease. Therefore, such conditions encompass those that directly cause premature ovarian

insufficiency (POI) such as bilateral benign ovarian tumours, severe recurrent endometriosis or genetic disorders (e.g., Turner's syndrome), and conditions that indirectly result in POI such as malignant or non-malignant diseases that require the administration of gonadotoxic chemo- or radiotherapy [6].

Whilst there is a tendency to focus primarily upon female infertility due to the intrinsically finite nature of female reproductive biology, it must be remembered that males are also distinctly susceptible to gonadotoxic agents, with one study reporting that up to 60% of male cancer survivors experience fertility impairment [7]. In fact, malignant diseases are amongst the most significant indirect contributors to infertility worldwide, with some of the most commonly-used classes of chemotherapeutics, alkylating agents, having been shown to induce POI in 42% of women treated [8]. The situation is further complicated by the widespread use of novel targeted therapies whose impact upon fertility is largely unknown [9]. These advances in cancer treatment efficacy (coupled with societal pressures to delay childbearing) have led to an increasing proportion of cancer survivors who wish to further add to their families, resulting in increased public awareness of treatment-induced subfertility, increased demand for fertility-preserving procedures, and the emergence of a brand-new field: oncofertility [10, 11]. This new discipline is badly-needed, providing patients with essential information that will impact upon their treatment decisions and future family planning, and aiming to disrupt the traditional lack of emphasis placed on iatrogenic infertility in the oncological sphere [11–13].

Another newly-emerging paradigm in gamete cryopreservation is its implementation as a timesaving method in fertility treatment. Age is the most significant determinant of IVF cycle outcome, meaning that older females who present for treatment may be considered for multiple consecutive rounds of ovarian stimulation and egg collection, thereby facilitating the freezing of large numbers of eggs which can later be fertilised and transferred [14]. This is a significant advantage for couples who may want multiple children, or who find the storage of a large number of embryos ethically questionable. It is open to debate whether this application should be considered medical or social, but as technology advances, it is important we consider such applications that lie within the 'grey areas' of medicine.

2.1. Particular considerations regarding the cryopreservation of spermatozoa

Sperm cryopreservation is the only established fertility preservation method in post-pubertal males, and has been in clinical use for over 50 years [15]. Its early adoption to the clinical realm is attributed to the accidental discovery of the cryoprotective properties of glycerol on sperm cells, their abundance for experimental uses and their small size. This latter property is an extremely important one, reducing the likelihood of damaging intracellular ice crystal formation during the freezing process. Whilst cryopreservation by slow freezing protocol was the first method used successfully, it causes extensive chemical and physical damage to sperm cell membranes, with only 60% of sperm regaining motility post-thaw [16]. Comparative studies have demonstrated that non-standard methods of rapid freezing (vitrification) using liquid nitrogen give better post-thaw motility rates and alter protein expression profiles less, as well as being more time- and cost-efficient [17, 18]. Whilst both methods result in

a significant reduction in viability, the (generally) high number of spermatozoa per sample means that lower survival rates are acceptable. As such, either cryopreservation method may be used effectively. This relatively low bar for post-thaw viability contrast hugely with oocyte cryopreservation, where the numbers of gametes collected tends to be small, and therefore more stringent protocols and attrition rates are required.

Sperm samples for cryopreservation are usually obtained by masturbation, but if in the cases of azoospermia, males who are unable to provide a sample (e.g., for psychosocial or physical reasons) or those who have previously undergone a vasectomy, surgical techniques may be employed. These include epididymis aspiration, testicular needle biopsy (TESE) or needle aspiration (TESA), with TESE having impressive success rates of 85%, even following chemotherapy for testicular cancer [19]. It is important to note that although these methods of sperm retrieval are effective, all require that the patient is able to produce spermatozoa, even at dramatically decreased levels. Options are extremely limited for patients whose Sertoli cells are non-functional, or pre-pubertal males, with the cryopreservation and autotransplantation of spermatogonial stem cells (SSCs) still classified as experimental, but showing promise in animal models [20]. In vitro maturation of SSCs, or SSC derivation from induced pluripotent stem cells (iPSCs) are also avenues under investigation [21].

Whilst both the American Society of Clinical Oncology (and almost all other) guidelines recommend that fertility preservation be offered to pubertal males before commencement of gonadotoxic treatment, only 25% of eligible males in the relevant cohorts bank sperm. These statistics are surprisingly low, especially when one considers the generally non-invasive nature of semen sample collection, and the wealth of prospective studies supporting that viewpoint that the overwhelming majority of men diagnosed with cancer wish to have children later in life [22]. One such study reported that 43% of patients surveyed ranked reproducing as a 'top 3' life goal [23]. It is therefore apparent that a disconnect exists in male fertility preservation that is not present to the same degree in the female equivalent. This may be due to routinely poor counselling by clinicians, but it is also possible that the priorities of young male patients may not adequately reflect their later life goals, or that male stoicism might affect the decisions made. Equally, the perceived high cost of cryopreservation and storage might have a role to play, even though robust cost-benefit analyses have shown sperm cryopreservation to be more cost-effective than post-therapeutic fertility management [19]. It has been evidenced that long-time storage does not seem to affect the fertilisation potential of sperm, as recently reported after 40 years of storage [24].

2.2. Particular considerations regarding the cryopreservation of oocytes

In contrast to spermatozoa, mature (MII) oocytes are large, fragile cells that are much more susceptible to water retention and ice crystal-mediated damage. Furthermore, addition of cryoprotectants may result in osmotic stress, with the cumulative effect of these stressors manifesting as thickening of the zona pellucida, premature cortical granule exocytosis and meiotic spindle disruption [25, 26]. Although this disruption of the meiotic spindle appears to be transient in almost all cases, there is robust evidence to show that cryopreservation negatively impacts oocyte gene expression and proteomics, with some cryoprotectants even shown to alter maternally-derived proteins which support early oocyte development [27–29].

The net result of this is a 'stressed' oocyte which is difficult for spermatozoa to penetrate and fertilise. As such, the clinical applications of oocyte cryopreservation were limited until the inception of the ICSI technique in 1992 [30], with the first pregnancy derived from frozen oocytes following in 1997 [31].

Another quantum leap forward in the efficacy of oocyte cryopreservation came with refinement of freezing protocols. Similar to the paradigm change seen in spermatozoa cryopreservation, vitrification (fast freezing) techniques were pioneered, first producing a live birth in 1999, and then being further improved by Japanese groups in 2003 [32, 33]. In contrast to the small increase in efficacy seen with the introduction of vitrification in spermatozoa cryopreservation, however, vitrification of oocytes seems to greatly increase post-thaw oocyte survival and fertilisation rates, with a 2014 Cochrane review finding a relative increase in oocyte survival of 29%, and a 19% increase in fertilisation [34]. An additional meta-analysis of three RCTs in 2016 reported a 16.1% increase in survival (RR = 1.23, 95% CI: 1.02–1.49; P = 0.031) [35]. The efficacy of the vitrification technique was further confirmed when a large prospective study of Spanish egg-donation programmes could not detect any statistically significant difference between using fresh donor eggs, when compared to vitrified frozen eggs [36]. It is important to note, however, that both of these techniques are inherently operator-dependent; with vitrification especially variable due to the need to complete the process within seconds [37]. This is an important caveat, and highlights the importance of training and upskilling, especially when considering the variable experience that operators may have within the same fertility clinic. It must also be clarified that the survival rates of oocytes (and the number collected) are likely dependent on the age and disease status of the donor, meaning that the extremely high survival rates of thawed oocytes reported by some studies on donor eggs (in excess of 96%), may not be truly representative for a significant proportion of patients who undergo fertility-preserving treatment [38].

It is clear, therefore, that the path to the clinic for oocyte cryopreservation has not been a straightforward one, with the early, highly-ineffective methods of oocyte cryopreservation making it an unrealistic and imprudent option for females in urgent need of fertility preservation, such as oncology patients. Cancer in reproductive age is twice as common in females as in males, and more than half of those diagnosed are expected to undergo treatment that compromises their fertility [39]. One large retrospective study highlighted this, indicating that whilst the incidence of treatment-related acute ovarian failure (AOF) was approximately 10%, these figures greatly misrepresent the total age-specific impact on fertility, with 40% of those not reporting AOF encountering infertility by the age of 35 [40]. Furthermore, the probability of early menopause was 'at least' 25% by age 30 [40]. It is likely, therefore, that effects on fertility may often relate to a reduction in the overall number of primordial follicles, and may therefore remain undetected until later in life. In a society where increasing numbers of women are choosing to delay childbearing, this may mean that women who are presumed to have normal reproductive activity following resumption of menstruation may not try to conceive as early as they are able to, and then later encounter difficulty.

It is important to consider that patients undergoing fertility-compromising cancer treatments may only have sufficient time for one round of ovarian stimulation and egg collection before their treatment must begin. This process of controlled ovarian stimulation (COS) followed by

egg collection generally takes approximately 2 weeks to complete, with patients able to start chemotherapy within 48 h of completion. Whilst concerns had initially been raised about the administration of such high doses of exogenous gonadotrophins to patients with hormone-sensitive cancers (e.g. breast, ovarian), effective and safe stimulation protocols using aromatase inhibitors have been developed and shown to result in no increased risk of recurrence in breast cancer, after a mean 5-year follow-up period [41]. In addition, the use of GnRH antagonist regimens (in place of the usual GnRH agonist regimens) allow ovarian stimulation to be started at any point in the menstrual cycle ('random-start protocols'), thereby minimising treatment delays. These GnRH antagonist regimens have been shown to result in the collection of similar numbers of mature oocytes and produce similar fertilisation rates [42]. Moreover, they have been shown to result in a lower risk of ovarian hyperstimulation syndrome (OHSS) than conventional protocols [43].

As such, refinements in cryopreservation techniques and stimulation protocols represent incredibly important steps for cancer (and elective) patients, increasing both the safety of oocyte collection and the likelihood of a live birth following completion of treatment.

2.3. Clinical outcomes of using cryopreserved gametes

As outlined above, the cryopreservation of gametes is a technically difficult and expensive process. As such, it is essential that the true success rates of these procedures be analysed using clinical endpoints, in order to prevent delays to treatment, unnecessary harm to patients and to disrupt the growing belief amongst the general proportion that egg freezing constitutes an infallible 'insurance policy' against age-related fertility decline.

In order to assess the success of cryopreservation we must first examine the parameters by which success is gauged. The most realistic way to evaluate the efficacy of cryopreservation techniques (and indeed individual clinics) is through the comparison of live births achieved per oocyte thawed. Although this may seem obvious, there is a growing propensity for some clinics (especially those who derive a significant proportion of their income from social egg freezing) to display these statistics in a manner that makes them appear more impressive. For example, some success rates might be represented using clinical pregnancy rates per thaw cycle; with some studies reporting this to be as high as 78% [44]. This figure is not an accurate representation of the reality faced by most patients, with the largest reported study of 3610 vitrified oocytes producing an oocyte survival rate of 90%, translating to a clinical pregnancy rate of 48% and an 'oocyte-to-baby' rate of just 6.5% [45]. If this same study were to be presented alternatively, it could be quoted as a delivery rate of 78.8% per oocyte donation cycle. As such, it is clear that there must be further efforts to homogenise how 'success' is calculated, and increased scrutiny of how these results are presented to potential patients. It is essential, also, to note that this data (and indeed almost all data on oocyte cryopreservation) has been generated from oocyte donation programmes. This is significant because oocyte donors tend to be carefully-selected, young individuals, whose eggs are likely to be of greater quality than the average patient wishing to engage in autologous fertility preservation. In fact, this viewpoint is supported by findings that only 32% of patients freezing their eggs were below the age of 35, and recent data showing reduced yield of oocytes collected in oncology patients versus matched controls [5, 38, 44]. As such, it is likely that the true likelihood of a successful

live birth for patients in these groups is significantly lower than the figures generated by current data. It is essential, therefore, that the increasing availability of data from non-donation sources be interpreted and used to validate the statistics that are currently quoted.

The largest study using data collected from outside of egg donation programmes was carried out by Cobo et al., who examined the reproductive success of 1468 women undergoing elective oocyte cryopreservation for non-oncologic reasons [46]. Their data clearly demonstrates the impact of age at freezing upon potential success, with those who froze at or before the age of 35 having a 53.9% likelihood of a live birth per ET, whilst those freezing at or above the age of 36 had a 22.9% chance. This viewpoint was echoed by a recent HFEA report, who described patient age at freezing as 'the most important factor', whilst age at thaw was not determined to have any statistically significant impact [5]. The same study also demonstrated the importance of the number of oocytes obtained to vitrify in increasing chanced of a live birth, with an increase from 5 to 8 oocytes producing the most significant increase in LBR (8.4% per oocyte if <35). Whilst an average 'oocyte-to-baby' ratio is omitted, it is estimated to be significantly lower than the 6.5% achieved in donor programmes. Whilst this is an interesting figure, it is likely that it does not provide as clear a picture of the factors that impact oocyte viability as that provided by age-bracket stratification.

Consequently, we can conclude that the number of viable oocytes available for fertilisation is a clear determinant of the likelihood of successful pregnancy. The technique used to freeze and thaw the oocytes retrieved is thus of the utmost importance, with a multitude of studies confirming the advantages provided by vitrification protocols, both in terms of post-thaw oocyte survival and reported pregnancy rate. In fact, multiple studies reported the clinical pregnancy rate (CPR) to more than double when compared to slow-freezing protocols [47, 48]. In addition, there is increasing scrutiny on the impact that the rate of warming can have on post-thaw oocyte survival and characteristics. In fact, Mazur and Seki reported oocyte survival >80% when ultra-rapid warming was carried out, even when using traditional slow-freeze protocols. Further expanding on this, they demonstrated that such methods could be used to reduce the concentrations of cytotoxic chemoprotectant required for the vitrification process [49]. Interestingly, a recent meta-analysis of five studies concluded that there was no significant difference between the fertilisation rates, embryo cleavage or pregnancy rates achieved when using fresh versus vitrified oocytes [50]. This viewpoint is supported by recent data supplied by the HFEA, who concluded that the birth rate per embryo transfer (PET) was rising to over 19%, and within 2% of the overall IVF birth rate PET [5]. In addition, multiple studies have demonstrated that the length of storage has no effect on pregnancy rates or outcomes [45, 51]. As such, it is reasonable to conclude that cryopreservation techniques have advanced to such a stage that significant future improvements in success rates will likely relate to methods of increasing the yield of oocytes collected per stimulation cycle, or in the methods used to select the embryos to be transferred.

The above discussion is necessarily focused on female gametes, as spermatozoa quality has traditionally been seen to be of less importance owing to the large number usually obtained per collection and their high survival rate. It is also worth noting that studies have found no correlation between sperm quality and disease stage in oncology patients [52]. In addition, the advent of ICSI has meant that even 'poor quality' sperm samples with low motility scores

can be used to produce a viable embryo. That said, it is almost certain that there exist intrinsic variations in spermatozoa quality that currently evade detection, driving increased research into how we select the sperm we use for fertilisation, both in the context of conventional IVF, and in fertility preservation. The artificial techniques discussed have abrogated the physiological selection methods inherent to the natural reproductive process, paving the way for a growing need for the 'unnatural selection' of favourable gametes via novel biomarkers or growth characteristics. Ongoing avenues of such research include the assessment of spermatozoal DNA fragmentation rates (although evidence is not yet conclusive), and promising future avenues such as the stratification of sperm quality via spectrophotometric analytical techniques such as Raman spectroscopy [53]. In fact, the latter method would allow andrologists to select spermatozoa on the basis of both their homeostatic and epigenetic context [54].

It follows that an essential aspect of any discussion on the clinical outcomes of gamete cryopreservation must be that of perinatal outcomes. It is often easy to rely on pregnancy rate as the sole benchmark of a successful preservation cycle, but serious consideration must also be given to whether the progeny created are morphologically, genetically and developmentally 'normal'. Reassuringly, a number of analyses, one of 165 pregnancies and another of 936 infants, have found a comparable incidence of congenital abnormalities in infants born following oocyte vitrification, conventional IVF and natural pregnancy [55, 56]. There is also a growing body of evidence, however, that IVF may trigger epigenetic disruption in the developing embryo, potentially causing the slightly lower birth weights observed amongst children born as a result of these techniques [57]. That said, it is also possible that these differences are related solely to the increased ages of the patients within the IVF cohort. A long-established relationship exists between increased parental age and genetic dysfunction, with increased maternal age being linked to abnormal meiotic spindle function, and therefore the induction of gross chromosomal abnormalities such as Trisomy 21 (Down's syndrome) [58]. Similarly, it has been shown that the higher prevalence of single point mutations seen in children born to fathers of more advanced age is attributable to the higher number of mitotic replications that these germ cells have experienced [59]. It is thought that this is a direct cause of the increased rates of neurodevelopmental disorders, leukaemias and stillbirths seen in this paternal cohort [60]. As such, although age has a strong positive correlation with adverse perinatal outcomes, no cryopreservation-specific (or indeed fertility treatment-specific) causal relationship has yet been reliably established.

In fact, the most common perinatal outcomes that are directly attributable to IVF are due to multiple pregnancies. These usually occur as a consequence of the transfer of more than one embryo, and may result complications such as premature birth, intrauterine death and conversion to caesarean section [61]. Whilst this, and complications associated with advanced maternal age, certainly remain considerations in the fertility preservation sphere, the patients concerned tend to have fewer options and less time to achieve a successful pregnancy, making the delivery of multiple children more serendipitous than it otherwise might be. Indeed, as the average age of childbearing increases (due, in part, to ART), it is arguable that discussion of such 'difficult pregnancies' will be of less future importance, as prospective patients will almost always opt to try to conceive in the face of an increased risk of poor perinatal outcome, instead of not attempting to conceive at all.

In conclusion, although the oocyte conversion rates discussed above might seem extremely poor at the outset, it must again be stressed that modern assisted reproduction technologies circumvent the physiological selection mechanisms that serve to ensure only the most viable gametes survive. Success rates using cryopreserved gametes are almost comparable to those achieved using fresh gametes, and therefore it is reasonable to expect the efficacy of both techniques to advance in parallel as our knowledge and gamete selection methods improve. The **Table 1** presents several methods currently used to improving gamete selection for cryopreservation.

2.4. Societal and ethical aspects of cryopreserving gametes

Although the technical aspects of gamete cryopreservation have been discussed at length above, one must also consider the societal and ethical impact of such procedures. Gametes are incredibly prized cells; holding the genetic information is required to produce related offspring for those at high risk of fertility disruption. Therefore, the conditions under which they are stored, the individuals permitted to handle them, access related information whilst they

Technique	Description	Evidence
Pre-freeze swim-up preparation (spermatozoa)	Traditionally, sperm selection via preparative techniques was undertaken post-thaw. There is increasing evidence, however, to show that such swim-up techniques should be performed before cryopreservation to produce the highest percentage of viable spermatozoa. It is theorised that cytokine release from immune cells that are inadvertently included in cryopreserved samples may damage spermatozoa quality, and that this could be avoided using these pre-freeze techniques. This viewpoint has been supported by data from recent trials.	Petyim et al. [111]
Rate of cooling	As evidenced by the aforementioned increases in gamete quality using vitrification techniques, the rate of cooling during cryopreservation is extremely important. As such, efforts have been made to dramatically decrease the volume of the solution in which oocytes are vitrified (now 0.1–2 μL). To facilitate this, specialised carriers have been developed, including both open and closed systems. Comparative analysis of these two categories of systems has demonstrated similar oocyte survival rates, but significantly increased cytoplasmic vesicle presence (and theorised reduction in quality) in oocytes frozen using the closed system.	Bonetti et al. [112]
Low-CPA protocols	Protocols that employ low concentrations of cryoprotectants have the potential to combine the positive aspects of vitrification and slow-freezing, without their respective associated disadvantages. Although such protocols have been impractically complex and time-consuming, recent advances in quartz micro-capillary techniques are showing promise.	Choi et al. [113]
Single-gamete analysis	Although not yet adequately optimised for clinical use, analysis of individual gametes has the potential to revolutionise how ART is carried out. The increasing need for artificial selection has meant that there is now increasing scrutiny on spectrophotometric and other non-invasive analytical techniques, some of which have been shown to provide adequate comparative analysis for oocyte quality and sperm DNA fragmentation rate. Whether this comparative analysis will be of clinical use, however, remains to be seen.	Davidson et al. [114] Sanchez et al. [115]

Technique	Description	Evidence
In-vitro maturation (IVM) of immature oocytes	IVM aims to increase the yield of oocytes available for cryopreservation through the obtaintion of additional M2 oocytes from oocytes that would otherwise be discarded. Although data shows that approximately 35% of IVM oocytes can produce cleavage-stage embryos when fertilised, and this method has been suggested to increase the efficacy of treatment cycles aimed at fertility preservation, there is currently insufficient data to support the systematic use of IVM techniques or the freezing of immature oocytes.	Oktay et al. [116] Phoon et al. [117]
Selection via DNA fragmentation rate	It is clear that a both vitrification and slow-freeze protocols produce DNA lesions, either via full or partial fragmentation. Although modern analytical techniques can quantify this fragmentation (and resulting apoptotic induction), they most commonly result in destruction of the gamete in question. As such, although they may provide valuable information on the quality of a particular sample, they do not provide a solution for the accurate selection of gametes which may prove more viable that their morphologically-normal counterparts.	Valcarce et al. [118]
CPA equilibration temperatures	Changing the equilibration temperature with CPA and increasing the sucrose concentration added have both proven to be effective strategies to improve oocyte survival and fertilisation rates, respectively	Borini et al. [119]

Table 1. Methods of improving gamete selection when employing cryopreservation techniques.

are stored, and the length of time which they can be stored for are of the utmost importance. Although legislative circumstances may vary from country to country, the HFEA permits storage of gametes or embryos for an initial maximum period of 10 years, with this being extended by 10 years at a time on a case-by-case basis up to a maximum of 55 years [61]. These limits are important to protect the wellbeing of prospective children, and to prevent the misuse of genetic material. Furthermore, as technological advances in genetics allow increasingly accurate prediction of phenotype and disease likelihood, it is likely that the genetic material contained within gametes will need progressively more stringent protection. An example of such measures includes the recently-enacted General Data Protection Regulation (GDPR), which legislates for the prevention of the misuse of such genetic data [62].

The societal effects of the growing popularity of cryopreservation must also be considered. More women than ever before are experiencing the ironic dichotomy of spending the vast majority of their reproductive years trying no ensure that they do not fall pregnant, but then finding themselves unable to conceive when they try to. As such, the landscape of this exploding field is increasingly commercial, providing increased funds to facilitate advances in treatment efficacy at the cost of advertising cryopreservative services as an insurance policy against age-related fertility decline. There is also a worrying increase in the number of companies offering 'social freezing' as part of their employee benefit packages. This is a trend that propagates the misinformed idea that social cryopreservation guarantees a later pregnancy, and serves to perpetuate the societal pressure placed on women to delay childbearing [63]. The cost of such procedures (if not covered by insurance or a third party) is also a valid consideration, with various cost benefit analyses finding contrasting conclusions on whether it is more, or less cost-effective to cryopreserve in one's mid-twenties and return to them at age 40,

or just to attempt conventional IVF at age 40 [14, 64]. Whilst this is an important avenue of discussion, the superior success rates provided by the cryopreservation route are likely to provide a superior chance of obtaining a live birth.

While the risks associated with childbearing at an increased age may have the immediate downstream effects of reducing the incidence of certain genetic aberrations, it is also important to consider knock-on effects which may not be immediately obvious. It is possible that widespread societal gamete cryopreservation could unearth harmful novel ART-mediated epigenetic alterations, or further promote the delay of childbearing age. Such effects would doubtless affect the composition of our society, and the manner in which it functions. Therefore, the future direction and regulations governing this area must be scrutinised to determine what should, and should not be permitted. This is a more complex ethical discussion that falls outside of the scope of this chapter, but should nonetheless be kept in mind.

3. Current status of embryo cryopreservation

Since the early days of in vitro fertilisation (IVF) 40 years ago, there have been remarkable advances in clinical and laboratory areas that have opened the door to different variants of standard IVF procedure [65, 66]. Improvements of ovarian stimulation protocols enable the collection of several mature oocytes, which associated with the improvement of the IVF techniques and optimization of embryo culture result in the obtention of a large number of embryos. Therefore, embryo cryopreservation was a necessary evolutionary step for IVF-treatments with the first pregnancy after transfer of a frozen-thawed embryo being reported in 1984 [67]. Since then, embryo cryopreservation has become a widely used technic in assisted reproductive technology (ART), allowing the preservation of the remaining embryos following a fresh transfer for future pregnancies and as a modern tool to reduce multiple births by encouraging patients to transfer a single embryo [1, 68]. Additional indications for embryo cryopreservation are the embryo banking for preimplantation genetic screening, elective deferred embryo transfer, when the patient is at risk of a hyperstimulation and for fertility preservation [66, 69]. Thus, embryo cryopreservation greatly increased the safety and efficacy of IVF treatments and enable the later use of all the embryos obtained from a single oocyte pick-up.

Over the years, cryopreservation methods, protocols and stage at time of cryopreservation have changed, improving embryo cryopreservation techniques. Consequently, the number of frozen-thawed cycles increased worldwide [66, 70] with similar or even higher pregnancy rates compared with the transfer of fresh embryo [65, 70]. In Europe, the last report generated from registers by the European Society of Human Reproduction and Embryology (ESHRE) stated that 154,712 frozen-thawed cycles were performed in 2013, increasing the overall life birth rate by 6% [71].

Herein we will resume the evolution of the embryo cryopreservation methods, stage at which cryopreservation is performed and give an overview of the perinatal outcomes of frozen-thawed embryo transfers.

3.1. Methods of cryopreservation applied to embryos

Since the first reports of pregnancy and delivery after transfer of frozen-thawed embryos in the earlies 1980s [67, 72] various protocols of embryo cryopreservation were introduced. They mostly differ from each other in the type and concentration of cryoprotectants, equilibration timing, cooling rates and freezing devices [35]. Regardless of the cryopreservation method used, the goal is to suspend embryos in time by cooling embryos from ambient temperature to −196°C [73]. Nowadays slow freezing and vitrification are the two principal approaches for embryo cryopreservation, although vitrification has become favored over the last decade [35, 74].

In slow-freezing protocol the temperature is decreased sufficiently slowly to allow the adequate cellular dehydration but also minimising the formation of intracellular ice. This is only possible through the use of a programmable freezing machine. With this method, the samples are first exposed to a quick cooling rate of 2°C/minute until they reach −7°C. Then extracellular ice crystal formation is induced manually (seeding) by touching the vial or straw with precooled forceps as far away from the embryos as possible. As consequence, more water leaves the embryo allowing cryoprotectants to enter. After the seeding, the temperature decreases slowly (0.3–1°C/minute) untill it reaches temperatures approximately −40°C and then rapidly to −150°C with a cooling rate of approximately 50°C/minute. The embryos are then stored in liquid nitrogen until use [35, 74].

Although the first pregnancies and birth were obtain with an embryo cryopreserved with dimethylsulphoxide (DMSO) as cryoprotectant [67, 72] births using other cryoprotectants, such as propanediol (PrOH) were soon reported [75, 76]. Since then, this has become the cryoprotectant more widely used in combination with sucrose for embryo cryopreservation by slow-freezing [35] (**Table 2**). The disadvantage of the slow-freezing method is the formation of ice crystals, increasing the risk of cell damage during thawing. Therefore, despite this method has being used for over 30 years in IVF laboratories and considered safe, since the concentrations of the cryoprotectants used to avoid ice crystal formation are low, another technique was developed to improve embryo cryopreservation—the vitrification.

	Slow freezing	**Vitrification**
Cleavage stage embryos	1.5 M PROH plus 0.1 M sucrose are included in the most commonly used protocols [74]	EG-based method was early proposed for vitrification of cleavage stage embryos [122]
		DMSO-based method initially reported the lowest survival rate [123]
		EG/DMSO/sucrose in open or close systems are the most commonly used cryoprotectants
Blastocysts	Glycerol and sucrose as cryoprotectants are included in the most commonly used protocols [74]	EG and DMSO were the cryoprotectants used in the first pregnancy reported after blastocyst vitrification [124]
		EG/DMSO/sucrose in open or close system are also used [97, 125]

Abbreviations: EG: ethylene glycol; DMSO: dimethylsulphoxide.

Table 2. Commonly used protocols for cryopreservation of cleavage stage human embryos and blastocysts.

With vitrification, the ice formation is almost eliminated since the cells and the extracellular milieu are solidified into a glass-like state [77]. This method has an extremely high cooling rate in the range of 2500–30,000°C/minute till −196°C by immediate exposure to liquid nitrogen [74, 77]. Despite the high concentrations of cryoprotectants that this method requires, its potential toxicity is reduced by the short time of exposure and the small volume of cryoprotectants used [35]. As in the slow-freezing method different cryoprotectants were tested, leading to the current preferred combination of DMSO (15%), ethylene glycol (EG-15%) and sucrose (0.5 M) in a minimum volume (≤1 μl) [78] (**Table 2**). The biggest difference between vitrification protocols relates to the cooling and storage methods employed, with open system, involving direct embryo contact with the liquid nitrogen, or closed system involving specific devices to avoid direct contact with the liquid nitrogen [35].

With vitrification a laboratory can expect to obtain an increased embryo cryosurvival rate comparing to the slow-freezing method (**Table 3**), has well as a beneficial effect in the clinical pregnancy rate and live-birth rate per embryo transfer [35, 74, 79, 80]. Additionally, vitrification method does not require any specific equipment and is less time consuming compared to slow-freezing. Consequently, many laboratories worldwide have completely replaced slow-freezing with vitrification [35].

3.2. Cryopreservation at cleavage stage vs. blastocyst

The first pregnancy was obtained with an embryo cryopreserved at eight cells stage [67]. Since then the procedure has changed several times, with the current practice being to preserve either at the cleavage stage Day 2 or 3 of culture or at the stage of blastocyst at day 5 or 6 of culture, despite no clear evidence of which strategy is more beneficial for frozen-thawed embryo transfer [81, 82]. Since only few randomised controlled trials (RCTs) have been conducted to determine what stage of development optimises cumulative birth rate for the retrieval cycle, most of the available data about the timing of embryo cryopreservation is derived from outcomes of fresh cycles [73].

Despite the method of cryopreservation and embryo's stage, the embryo selection for cryopreservation is based on their morphology and pre-freezing morphology is directly related with cryopreservation success and efficiency [83].

	Cleavage stage		**Blastocyst stage**	
	Day 2	**Day 3**	**Day 5**	**Day 6**
Morphology before cryopreservation [74, 81, 97]	≥4 blastomeres <25% fragmentation No multinucleate blastomeres	≥6 blastomeres	Blastocysts are scored according to expansion, inner mass and trophectoderm using Gardner scoring system – 3BB or better	
Morphology after thawing [74, 120]	≥50% intact blastomeres Higher number of blastomeres after 24 h of culture		Scoring according to Gardner, as before cryopreservation	
Expected survival [74, 121]	61.4–87.5% with slow freezing 64–94% with vitrification		76.3–88% with slow freezing 84–100% with vitrification	

Table 3. Morphological aspects of embryos before/after cryopreservation and expected cryosurvival.

For cleavage stage embryos, it is recommended that the embryos selected for cryopreservation should have 4 cells at day 2 and 8 cells at day 3, less than 10% of fragmentation, stage specific cell size and no multinucleate blastomeres [84] (**Table 3**). After thawing, embryos with 100% intact blastomeres will have a higher implantation. However, in embryos with 50% or more cells intact post-thaw and with mitotic resumption, the number the cells at the transfer may be more predictive of the embryo's ability to implant than the percentage of cells surviving at the time of thawing [85–87]. These parameters remain the most clinically important criteria to evaluate the implantation rate potential till today [83].

Advances in culture systems have made possible to prolong embryo culture until the embryo reaches the blastocyst stage. Thus, over the last decade blastocyst transfer at day 5/6 of culture has greatly increased and is seen for some as a "natural selection" of the most viable embryo, similar to the process during spontaneous conception [88, 89]. However, the clinical efficacy of blastocyst transfer over cleavage stage transfer is debatable. In fact, in 2016 a Cochrane meta-analysis reported an increase of clinical pregnancy and live birth after blastocyst transfer [82] but 1 year later another meta-analysis did not find any statistical difference in outcomes when comparing the transfer of embryos at the cleavage stage or blastocyst [90], the same results were previously described for cryopreserved embryos [81].

As with cleavage stage embryos, assessment of blastocyst stage cryopreservation outcomes requires attention to variety of factors before cryopreservation and after thawing in addition to methodology. Outcomes have been shown to be dependent on pre-freeze quality of the blastocyst and time required to reach the blastocyst stage [91, 92].

The most commonly used blastocyst grading systems assigns scores to three morphologic aspects of the embryo: quality of inner cell mass—grades A, B, C; quality of the trophectoderm—grades A, B, C and degree of expansion (**Table 3**). Blastocyst score at the time of cryopreservation was associated with survival and implantation rates [93–95]. Other factors that may contribute to outcomes are the day the cryopreserved embryo reached the blastocyst stage [5, 6], whether the blastocoel was collapsed or not prior to cryopreservation, and evidence of blastocoel re-expansion prior to transfer [96, 97].

3.3. Clinical and perinatal outcomes of pregnancies achieved using frozen-thawed embryo transfers

Frozen-thawed cycles increased during the last decade and a concern about the perinatal outcome also have risen [66]. Although embryo cryopreservation is a well established procedure, long-term studies are still sparse [35]. Data are reassuring suggesting that pregnancies obtained from frozen embryos are not associated with an increased perinatal risk compared to fresh transfers [98–104]. Several reviews have indicated a slightly better result when frozen-thawed embryos were used compared to the fresh transfer, with reduced risks of preterm birth, small for gestational age babies, low birth weight babies and pre-eclampsia,

which could be justified by the endocrine milieu of the stimulation when the transfer is made fresh [70, 99, 102, 104].

Furthermore, a systematic review, published recently, confirmed that singleton babies born after the transfer of frozen-thawed embryos have higher weight at birth when compared to babies born after the transfer of fresh embryos, as well as a higher risk of hypertensive disorders during pregnancy [70].

3.4. Future perspective: the freeze-all strategy

To further improve IVF outcomes, it has been suggested to freeze all the embryos obtained in a stimulation cycle and then plan a deferred transfer during a natural cycle or with hormone replacement with exogenous estradiol (E2) and progesterone (P) for endometrial priming [105, 106]. With this strategy, the frozen-thawed embryos are transferred into a more "physiological milieu" which seems to improve implantation and outcomes compared to fresh transfer [106–108]. However, almost all the data was obtained in patients with high ovarian response patients and thus it was suggested that the freeze all strategy should be perform on patients with a risk of ovarian hyperstimulation syndrome (OHSS), since it was not clear if normal and poor responders will be the same benefits from freezing all the embryos [109].

A recent retrospective study using the general population has reported that 50.74% of patients using the freeze-all strategy achieved a live birth after the first complete cycle [105]. Additionally, another study indicated positive results in poor ovarian responders, and suggested the freeze-all strategy as an alternative to cycle cancellation for these patients [110]. Despite these positive results, large multi-centre randomised controlled trials are needed to evaluate the freeze-all strategy [105, 110].

4. Conclusion

In conclusion, the cryopreservation of gametes and embryos is a rapidly developing field that demonstrates increasingly comparable success rates to those encountered in conventional IVF using fresh gametes or embryos. Aiming to provide reproductive autonomy for patients, it is intrinsically intertwined with both societal and ethical issues, and will doubtless play an increasingly central role in how we as a species reproduce over the coming decades. Research indicates also safety of reproductive treatments using cryopreserved gametes and embryos.

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Author details

Max Waterstone[1,2], Amandine Anastácio[1,3] and Kenny A. Rodriguez-Wallberg[1,3]*

*Address all correspondence to: kenny.rodriguez-wallberg@sll.se

1 Department of Oncology-Pathology, Karolinska Institutet and Laboratory of Translational Fertility Preservation, Cancer Center Karolinska (CCK), Stockholm, Sweden

2 Faculty of Medicine, Trinity College, Dublin, Ireland

3 Department of Reproductive Medicine, Division of Gynecology and Reproduction, Karolinska University Hospital, Stockholm, Sweden

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Chapter 4

Cryopreservation of Platelets: Advances and Current Practice

Miloš Bohoněk

Additional information is available at the end of the chapter

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Abstract

Conventional fresh platelets stored at 20–24°C have a short shelf life, at most 7 days. Their main disadvantage is logistics as it is more difficult. This limitation is especially problematic for emergency and intensive care departments for managing massive bleeding. The early and aggressive use of blood products for massive hemorrhage may correct coagulopathy, control bleeding, and improve outcomes. The timely availability of platelets at the shortest time after the injury is often problematic. Many hospitals cannot afford to have platelets permanently in stock because of its short shelf life. Cryopreservation and storage of frozen platelets may significantly prolong their shelf life. Thus, frozen platelets provide long-term accessibility in situations where fresh products are not available. The most widely used method for the platelets cryopreservation is freezing at 5–6% DMSO at −80°C. The production of cryopreserved platelets is not technologically demanding, they can be easily thawed and reconstituted. Frozen platelets are an alternative blood product for urgent orders in connection with heavy bleeding. They are cost-effective functional platelets product for the management of bleeding and should be considered for wider use in clinical practice, such as autologous platelets, rare or HLA/HPA compatible platelets and platelets for non-transfusion use.

Keywords: cryopreservation of platelets, frozen platelets, DMSO

1. Introduction

Various methods of cryopreservation of blood cells are generally known and have been used for a long time. The storage of blood in the frozen state presented one of the alternative ways of storing blood components; this possibility was intensively explored in the 1950s and 1960s, when the shelf life of nonfrozen red blood cells did not exceed 21 days at those times. This

time limitation significantly reduced flexibility of usage of RBC products and contributed to their dramatically high and wasteful expiration reaching up to 30%. The short shelf life of the RBCs resulted in the transfusion services not being able to meet demands of quickly evolving surgical disciplines, particularly cardiovascular surgery and radical surgical oncology. In military and emergency healthcare, utilization of these 3-week products as a way of creating blood supplies was even more complicated, almost unthinkable. The storage of frozen red blood cells therefore presented a great prospect [1].

Nevertheless, some areas with a need to long-term storage of the blood cells still remained—for example, being the military transfusion, emergency transfusion service, storage of rare blood cells, or special autologous transfusion programs. Blood substitution and blood supply are permanent strategic and logistic problems of the military medical services across the world arising from the blood, has a limited shelf life and need the special transport and use conditions. The same problem must solve the national healthcare authorities in programs of the national blood crisis policy, where to get a huge amount of blood supply any time at any place in the case of disaster, terrorist attack, and war. The therapeutic problems in immunohematology cases can solve by stock of rare blood, storage of autologous blood for patients with rare erythrocyte or platelets antigens, and storage of autologous blood for patients with red blood cell alloantibodies or HLA/HPA platelets refractoriness with no chance to use common blood. All mentioned demands highly correspond with stock of frozen blood. New global security risks exalt this problem to all-society relevancy [1].

If the short storage time and shelf life can be problematic at fresh red blood cells, this disadvantage is greatly enhanced at standard platelet products. Fresh platelet, stored at a temperature of 20–24°C, have shelf life of 5–7 days. This excludes the production of larger supplies and makes their production, distribution, and use, logistically more difficult. This is particularly limiting for trauma centers, urgent hospital admissions, and intensive care units dealing with massive bleeding. Extremely difficult is the implementation of platelet transfusion in war medicine, remote areas, and pre-hospital care. Uncontrollable bleeding is the second leading cause of death in trauma patients. In battlefield casualties with severe blood loss, platelets are often deficient because of blood loss and because the platelets get consumed during blood clotting.

In many instances, frozen platelets are given prophylactically and autologous or HLA/HPA compatible frozen platelet transfusions have become an important part of the supportive care of leukemic patients at this institution during maintenance and reinduction therapy, when alloimmunization is frequently present.

2. Methods of cryopreservation of blood cells

The primary role of cryopreservation is the long-term preservation of cells and tissues while at the same time protecting them from the undesirable effects of frost. Already in 1866, Pouchet first described that frozen erythrocytes are destroyed after thawing [2].

During the changing process of aqueous solutions into solid state, it is water that changes its state of matter first. Water crystals are created from pure water, while the space between them is filled with concentrated electrolyte. This leads to cellular dehydration and to the pH change and those mechanisms destroy cell membrane before mechanical injury is caused by ice crystals [1, 3, 4].

Protection of cells from freezing is achieved by adding cryoprotective substances. Since these cryoprotectants usually cause a significant increase in osmolality, it is nevertheless necessary to have all the procedures monitored, and to have osmotic changes under control, in order to avoid an irreversible damage to cellular structures and membranes caused by them [1].

Mainly, **intracellular (penetrative) cryoprotectants**, as glycerol and dimethyl sulfoxide (DMSO), are used for the cryopreservation of blood cells. These substances penetrate the cellular membrane and do not present any toxic danger to the cell when in low concentration. Glycerol is used for red blood cells cryopreservation and DMSO for platelets freezing. The mechanism of the effect of penetrative cryoprotectants has not been fully clarified yet. Initially, the damage of cells was associated with the effect of ice crystals only. Cryoprotective substances, nevertheless, besides limiting the creation or frozen crystals, also modify these crystals' shape and size, and by changing their ionic ratio intracellularly and extracellularly; they also eliminate the damage caused by osmotic shock, which otherwise occurs during freezing. During the freezing process, penetrative cryoprotectants increase output of intracellular water, maintaining the osmotic balance in a partially frozen extracellular solution in this way. It results in not only reducing the cells' volume but also in the reduction of the osmotic load [1, 5–19].

Extracellular (nonpenetrative) cryoprotectants are macromolecular substances and due to their molecular mass, they do not penetrate cellular membrane and are mostly used for rapid and ultra-rapid freezing. The mechanism of the nonpenetrative cryoprotectants effect lies in their ability to stabilize cellular membrane and also in so-called vitrification. When there is water (with temperature below 0°C) turning into ice, nonpenetrative cryoprotectants remain outside the cells, where they secure the creation interspaces between cellular membrane and extracellular environment. Electrolytes segregated from freezing solutions are being concentrated in these interspaces [1].

This cryoprotectans, previously rarely used, are no longer used for cryopreservation of blood [20–24].

3. Cryopreservation of platelets

3.1. Methods of platelets cryopreservation

Platelets may be frozen using various types of cryoprotectants: intracellular (DMSO and glycerol) or extracellular (HES and dextran). HES and dextran were found to be poor

cryoprotectives, PLTs cryopreserved in glycerol gave lower yields and poor in vitro viability compared with those cryopreserved in DMSO, which is most suitable cryoprotectant for platelets.

Djerassi et al. [25] were the first to report on the use of 5% dimethyl sulfoxide and cooling at 1°C/min for successful cryopreservation and transfusion of human platelets. To avoid the effects related to the cryoprotectant itself (e.g., nausea, vomiting, local vasospasm, garlic-like taste, and body odor), Lundberg et al. [26] have introduced a post-thaw washing step. A method used by Schiffer et al. [27] has become the "standard" method for this purpose.

In 1956, Klein et al. reported the use of previously frozen platelets in an actively bleeding thrombocytopenic patient and ever since numerous studies have been reported on both the in vitro and in vivo efficacies of cryopreserved platelets [28]. Since Schiffer et al.'s 1976 study on the use of autologous platelets for the treatment of patients with leukemia, relevant studies until 1990s showed that the platelets were damaged to a significant extent by the freezing process that decreased their efficacy when compared to fresh platelets [27]. These results were supported by other in vitro studies that assessed the platelets' primary hemostatic functions. It has been demonstrated that the in vivo hemostatic functions of cryopreserved APCs were superior to the fresh preserved platelets [29] and reported the procoagulant changes in the frozen-treated platelet membrane surfaces [30].

The most widely used method for the platelets cryopreservation is freezing at 5–6% DMSO concentration at −80°C, with their storage at −65°C and lower. The method of platelet cryopreservation using DMSO was developed in the 1970s by Robert Valeri with the support of the US Navy's research program as a possible substitute for native platelets for transfusion therapy for wounded service personnel during military operations [19, 29, 31]. In the original method, the DMSO and the supernatant needed to be washed out upon thawing, making the method arduous for use in field hospitals. Over time, the procedure was adjusted to remove the excess DMSO and supernatant prior to freezing [29, 32, 33]. The method is simple, inexpensive, and requires no special equipment. For transport over long distances, transport containers filled with dry ice are used. Alternatively, it is possible to use transport active freezers providing a temperature of <−65°C. As the storage temperature of DMSO-cryopreserved platelets ranges from −80 to −65°C, the use of liquid nitrogen (or its fumes) is not necessary, and mechanical deep freezers can be used for storage instead. After thawing, the platelets are suspended in thawed plasma and there is no need to wash out the cryoprotectant. Original method can be modified by resuspension of thawed platelets in saline (0.9% NaCl) or plasma additive solution (PAS).

The procedures **for cryopreservation of platelets** according to the modified Valeri method are as follows:

I. To the collecting container with standard unit with apheresis or buffy-coat pooled platelets, preferably leucodepleted, with >280 × 10^9 PLT/unit and in the original donor plasma, is added 75 ml 25% solution DMSO in 0.9% NaCl resulting to 5–7% final DMSO concentration in platelet unit (**Figure 1**).

II. The container with PLTs + DMSO is connected with smaller container (using sterile connection device), where platelets will freeze (**Figure 2**).

III. Centrifugation 20 min, use soft spin (**Figure 3**).

IV. Gently removing of supernatant using the manual extractor and using visual control, tube sealing and labeling. The final product has 13–15 ml (**Figures 4** and **5**).

V. Freezing in cartoon box at −80°C, storage at −65 to −80°C (**Figures 6** and **7**).

The procedures for **thawing the platelets** according to the modified Valeri method are as follows:

I. One unit of frozen platelets and one unit of frozen plasma are thawed to 34–36°C. It is recommended to check the concordance of temperatures of both products at surface temperature, using contactless infrared thermometer (**Figures 8–11**).

II. The thawed platelets are gently "spreading" using a gauze with emphasis to flatten of potentially aggregates (**Figure 12**).

III. Connecting container with thawed platelets and container with thawed plasma, using sterile connection device. The plasma is transferred into the platelets by gentle stirring. Transferring the contents of the bags back and forth (three times) ensures a perfect mix of the products and a homogeneous suspension is obtained (**Figures 13** and **14**).

Note, the visual control is focused mainly on the presence/absence of aggregates. In thawed, previously frozen platelets, usually is not seen swirling phenomenon. The explanation is, that thawed thrombocytes are activated, altered in shape, with numbers of pseudopodia on the surface that make this optical phenomenon impossible.

IV. The final product (in the original platelet container) is detached from the plasma container, labeled and released for transfusion. The thawing and reconstitution does not exceed 30 min.

V. If plasma is used for reconstitution, as described above, the type AB plasma mixed with type O platelets is used. However, for reconstitution, other solutions can be used, PAS or saline.

VI. The shelf life of thawed and reconstituted platelets depends on the technical procedure of adding DMSO. It is usually 6 hours because of a non-sterile connection of a glass bottle with DMSO.

3.2. Features and quality of cryopreserved platelets

Platelets stored frozen are efficient in primary hemostasis after thawing. They efficiently contribute to stop bleeding as a part of complex transfusion therapy or damage control resuscitation in polytrauma patients and patients with massive bleeding. Some studies confirm that after reconstitution, the life span of platelets cryopreserved using DMSO in human circulation is comparable to native platelets in vitro [29, 34–37].

Figure 1. Add DMSO to collected fresh platelets.

Figure 2. Sterile connection of the container with PLTs contained DMSO to container for freeze.

The most widely used method is the reconstitution of cryopreserved platelets in thawed plasma, but there seems to be no significant difference between platelets reconstituted in other solutions, such as saline or PAS [29, 38, 39].

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Figure 3. A large volume centrifuge for platelets centrifugation.

Figure 4. Removal of supernatant from platelets before freezing using the manual extractor.

Although the platelets stored by cryopreservation are efficient in hemostasis, they are affected by a number of functional defects during storage and preparation for transfusion. The process of freezing and thawing causes changes in platelet morphology and affects their function. Approximately 15% of cryopreserved platelets lost surface-bound GPIb, while there was no measurable loss of GPIIB/IIIa during cryopreservation. The

Figure 5. Removed supernatant (left) and the final platelets product prepared for freezing (right).

Figure 6. Platelets before freezing.

cryopreserved platelets also showed a significant decrease in aggregation to ristocetin, but no loss of response to the stronger agonist, thrombin. Even though these defects are of a minor clinical relevance and the cryopreserved platelets were shown to be safe and

Figure 7. An example of −80°C portable freezing box.

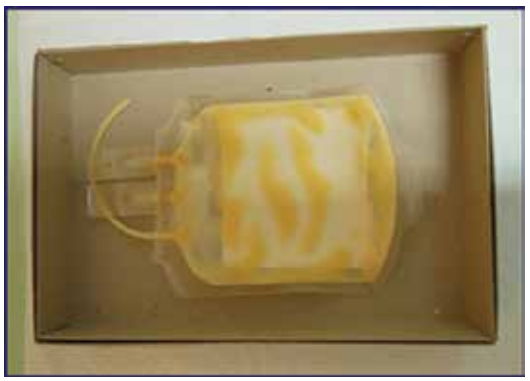

Figure 8. Frozen platelets.

effective for the treatment of abnormal bleeding, it is still necessary to reckon with these changes [22, 40, 41].

In cytometric observation, the frozen platelets contain about 85% of the particles in the microparticle area and only about 15% of the particles in the platelets region. For fresh platelets, this ratio is about 20% of microparticles and 80% of platelets. The question is to what extent higher amounts of microparticles are responsible for the observed higher hemostatic efficacy

Figure 9. Frozen plasma.

Figure 10. Plasma/platelets thawer.

of cryopreserved platelets. Additionally, cryopreserved platelets are considerably smaller than fresh platelets and have a lower perpendicular light scattering, reflecting not only their smaller size but also their spherical shape. Unlike the fresh platelets, frozen platelets are highly positive for Annexin V binding. This may contribute to their higher thrombin generation potential and lower circulating ability [42].

Platelet cryopreservation is associated with the release of platelet membrane particles and thrombin generation. The microparticles formed by cryopreservation carry phosphatidylserine on their surfaces and thus are phenotypically different from those found before freezing. Cryopreserved platelets have greater endogenous thrombin potential than fresh platelets [43].

Figure 11. Temperature check.

Figure 12. Spreading of thawed platelets.

This confirms the fact that platelet activation release of substances that potentiate the growth of thrombin generation occurs during cryopreservation and subsequent reconstitution. This is a set of reasons why the frozen platelets exhibit increased coagulation activity leading to faster clot formation with a concurrent decrease in clot strength. Such fact is confirmed by the thromboelastography measurements. The TEG curves evidently show a decrease in coagulation initiation time, that is, higher coagulation activity and faster clot formation (wider angle α), and a reduced maximum clot strength (MA), which is, however, still sufficient for initial coagulation [44–46]. The coagulation activity is further increased by reconstitution in frozen plasma. Platelets resuspended in such a way are more efficiently coagulative than, for example, platelets resuspended in additive solution.

Some observations by electron microscopy show plasma membrane disruption and vesiculation in 60% of thawed platelets. More than half of cryopreserved platelets exhibit signs of platelet membrane damage with a significant increase in its fluidity, induced by 6% DMSO alone and by the freezing and thawing process.

To speed up cryopreserved platelet reconstitution in plasma, it is possible to use plasma stored at a temperature of 4°C, meaning that the products can then be used within 15 min. However, for patients requiring the platelets for an indication other than hemostasis, resuspension in additive solution may be a suitable choice.

Figure 13. Sterile connection of thawed platelets with thawed plasma.

Figure 14. Transfer thawed plasma to thawed platelets.

The administration of cryopreserved platelets usually is not followed by any increase in blood platelet count, as in the case for fresh platelets and corrected count increments for platelets (CCI) is hard to use for evaluation of treatment effectiveness. This may be due to several causes. First is the broken structure and shape of thawed platelets associated with the higher amount of phosphatidylserine on their surface, which apparently contributes to the lower survival time

in circulation and lead to their immediate consumption in hemostasis. Another reason may be their more difficult resolution for the blood count analyzers. In the group of patients transfused with fresh platelets, a significantly higher platelet count was found in peripheral blood when compared to the patients transfused with cryopreserved platelets. Other laboratory and clinical parameters (clinical efficacy) are comparable. However, this is one of the reasons why cryopreserved platelets are recommended mainly for substitution in conditions associated with severe bleeding, and less for prophylactic treatment of hematological thrombocytopenia.

It is also necessary to keep in mind the differences associated with cryopreserved platelets, such as membrane-bound coagulation factors V and X, increased formation of thromboxane B2, and the significant presence of the released microparticles. However, these observations are unlikely to have great clinical relevance for the use of cryopreserved platelets in the treatment of massive bleeding, particularly in terms of any possible influence on their coagulation activity [47]. Similarly, clinical efficacy is not influenced by decreased platelet counts in the preparation or lower recovery rate after thawing. These are adequately compensated by a comparable life span in the patient's circulation, as shown in healthy volunteer studies published previously.

In the studies published so far, no severe reactions were reported following administration of frozen platelets and this was confirmed by our observation. Furthermore, no negative effect of increased coagulation activity of the cryopreserved platelets was observed [36, 48].

3.3. Use and perspectives of cryopreserved platelets

Cryopreservation and storage of frozen platelets may significantly (or unlimitedly) prolong their shelf life. Thus, frozen platelets provide long-term accessibility in situations where fresh and native products are not available and there is no way of obtaining them. The production of cryopreserved platelets is not technologically demanding, and furthermore, they can be easily thawed and reconstituted.

Early massive and complex transfusion therapy, excluding erythrocyte substitution, contributes significantly to coagulopathy correction and the alleviation of bleeding. The rapid administration of the whole spectrum of transfusion products is proven to have a positive impact on patient survival. Therefore, most current transfusion protocols and hemostatic resuscitation procedures are based on the co-administration of erythrocytes and plasma, supplemented with platelet transfusion [29, 49].

Platelet availability for the timely application of a modern massive transfusion protocol during the first "golden hour" following the onset of bleeding is often problematic. Furthermore, most hospitals cannot afford to have native platelets permanently available due to their short expiration and high price. Even university hospitals and large trauma centers may experience a limited availability of platelets in cases of urgent need for large quantities. Ensuring the availability of platelets in field military hospitals, namely in current international military operations, is even more problematic.

Although the given thawing and reconstitution procedure describes the use of a sterile bag tubing welder when connecting the platelet and plasma bags, the use of a sterile connection device is not necessary. In blood banks that do not have this technology, it is advantageous to connect the bags using simple tubing with spikes at both ends.

The relatively short shelf life of cryopreserved platelets (2 years) as blood product is based on the European directive and has no real evidential basis. The data from a study conducted in a laboratory at the Militaire Bloedbank in Leiden, the Netherlands, are currently being processed [50]. According to the preliminary information, these data support the possibility of extending the shelf life to at least 4 years. The preparation shelf life of 6 h, after thawing and reconstitution, is based on the fact that the process of adding the DMSO before freezing does not take place in a completely closed system and at the same time, it allows the use of tubing to add the resuspension media. If the DMSO was commercially manufactured in a plastic bag as a medical device using a sterile welder, it would be possible to extend the shelf life of the cryopreserved platelets even after reconstitution.

In recent years, there has been a relatively large renegotiation of interest in cryopreserved platelets as a promising blood product, which is being used, tested, and validated in a number of countries and institutions.

Therefore, cryopreserved platelets constitute a suitable alternative, which has been used by the Dutch Military Health Service and other countries for some time [4, 50–54]. The easy availability, compatibility, safety, and efficacy of the cryopreserved products significantly improved survival rates of patients with war injuries treated during international missions at Dutch army field hospitals in 2001–2012. Another advantage is the possibility of storing HLA-/HPA-matched platelets and rare platelets, as well as autologous platelets.

3.3.1. A brief overview of the use of cryopreserved platelets in the world

See Refs. [48, 51–53, 55–57].

3.3.1.1. Australia

Production:

Yes, for the military and for clinical civil studies.

Use:

In civil society so far only in clinical trials.

Method:

In 6% DMSO, 24–48 h after storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution at 30°C, exp. 6 h.

Advantages:

Long service life.

Decreased expiration and thrombo-thawing on the patient.

Increased procoagulant activity—an advantage for pac. with severe bleeding.

3.3.1.2. Belgium

Production:

Yes, for the army.

Use:

In civilian, not in a prospective military operation.

Method:

In 6% DMSO.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution, exp. 6 h.

Advantages:

Long shelf life, protrobotic potential.

3.3.1.3. Brazil

Production:

Currently no, recently for studies.

Use:

Not yet, prospectively.

Method:

In 6% DMSO.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution, exp. 4 h.

Advantages:

Long shelf life, immediate availability, the ability to store rare platelets.

3.3.1.4. Czech Republic

Production:

Yes.

Use:

Yes, so far Military University Hospital Prague, prospective in trauma centers.

Method:

In 6% DMSO, within 2 h of collection.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution at 32°C, exp. 6 h.

Advantages:

Immediate availability, procoagulant activity—suitable for severe bleeding.

Stock of platelets for field military medical service.

3.3.1.5. France

Production:

Yes—from 2015.

Use:

Yes: rare HPA/HLA platelets, severe bleeding.

Method:

In 5% DMSO.

Storage at −65 to −90°C, exp. 2 years/−180°C, exp. 3 years.

Plasma reconstitution at 32°C, exp. 6 h.

Advantages:

Platelets availability when native platelets are not available, platelets used for field military care and in remote areas.

3.3.1.6. Canada

Production:

Currently no, it is considered to be produced in the military blood transfusion service.

Use:

Not yet.

Method—considered:

In 6% DMSO.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution/PAS.

Advantages:

Long service life.

Procoagulant activity—suitable for severe bleeding.

Thrombocyte assurance in remote areas.

3.3.1.7. China

Production:

Yes.

Use:

Heavy bleeding (obstetrical bleeding), autologous platelets.

Method—considered:

In 5–6% DMSO.

Storage at −65 to −90°C.

Advantages:

Availability, hemostatic effect.

3.3.1.8. Poland

Production:

Yes.

Use:

Yes: 11–13,000 T.D./year (10–12% of total platelet consumption).

If there are no native, special indications: neonates and intranasal transfusions at immunological thrombocytopenia, HLA/HPA rare platelets.

Method:

In 5% DMSO, within 24 h of collection.

Storage at −65 to −90°C, exp. 1 year/−140°C, exp. 2 years.

Reconstitution in 0.9% NaCl, exp. 2 h at 20–24°C.

Advantages:

Immediate availability, possibility of provision of HLA/HPA rare platelets.

Long service life.

3.3.1.9. Singapore

Production:

Yes—only for research yet.

Use:

No, they are looking forward.

Method:

In 6% DMSO, from BC.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution at 32°C, exp. 4 h.

Advantages:

Long shelf life, total expiration and destruction of platelets, platelet collapse in the absence of native.

3.3.1.10. Spain

Production:

Yes.

Use:

Yes, HLA typed platelets.

Method:

In 6% DMSO.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution at 32°C, exp. 4 h.

Advantages:

Providing HLA typified thrombocytes.

3.3.1.11. The Netherlands

Production:

Yes, only in military blood bank.

Use:

Yes, only in the field military health service.

Method:

In 6% DMSO, from apheresis, 24 h after collection.

Storage at −65 to −90°C, exp. 4 years.

Plasma reconstitution at 32°C, exp. 6 h.

Advantages:

Providing comprehensive hemostatic therapy in field military health.

3.3.1.12. Russia

Production:

Yes.

Use:

Cardio surgery.

Method:

In 5% DMSO.

Storage at −65 to −80°C, exp. 2 years.

Plasma reconstitution.

Advantages:

Maintain a stock of platelets, platelets HPA/HLA compatible.

3.3.1.13. Turkey

Production:

Yes

Use:

The strategic location of Turkey mandates governmental medical organizations in establishing frozen platelet and erythrocyte stocks.

Method:

In 4–6% DMSO.

Storage at −65 to −80°C, exp. 2 years.

Plasma reconstitution/0.9% NaCl.

Advantages:

Maintain a stock of platelets, military use.

3.3.1.14. USA

Production:

Yes, so far only for studies, in perspective after FDA approval.

Use:

Not yet, only in studies.

Method:

In 4–6% DMSO.

Storage at −65 to −90°C, exp. 2 years.

Plasma reconstitution/0.9% NaCl.

Advantages:

Maintain a stock of platelets and stock of HLA-typed platelets, military use.

4. Conclusion

The cryopreservation of blood is a method, which solves various problems in blood transfusion service. The main application is in military medicine and blood crisis policy, but also in special transfusiology fields, such as the storage of rare red blood cells and long-term storage of autologous blood. Due to modern procedures, which allow for prolonged shelf time after thawing and reconstitution of frozen blood, the use of frozen blood is now more flexible and less limited. Cryopreserved blood products are fully in comliance with European legislation [1].

Cryopreserved platelets have all the necessary prerequisites to constitute a product suitable for a possible wider application. Due to the extended shelf life, it is possible to create sufficient supplies of these transfusion products without a substantial cost increase. Cryopreserved platelets are suitable for both civilian and military use, particularly for the treatment of acute conditions associated with massive bleeding, when no permanent or sufficient supply of fresh platelets is available. Cryopreserved platelets act as a substitute for human platelets by helping the blood clotting mechanism in patients, who have a deficiency of platelets. Thawing and reconstitution is a simple procedure that takes no more than 30 min.

Cryopreserved platelets may find use in other indications, such as the autologous products, rare or HLA/HPA compatible platelets or in a wide range of nontransfusion applications. Due to their low production cost, the use of cryopreserved platelets does not represent a significant increase in the cost of transfusion therapy. Our work and previous studies suggest that cryopreserved platelets are efficient, effective, and safe.

Despite the stated advantages, the use of cryopreserved platelets in clinical practice has hitherto been rather limited and scarce. Their wider application is hindered by relatively little data on their in vitro attributes or on the comparison with the attributes of fresh platelets, and the complete absence of clinical studies evaluating their efficacy in vivo.

Author details

Miloš Bohoněk

Address all correspondence to: milos.bohonek@uvn.cz

Department of Hematology and Blood Transfusion, Military University Hospital Prague, Praha, Czech Republic

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Chapter 5

Cryopreservation of Preantral Follicles

Ellen C.R. Leonel, Carolina M. Lucci and
Christiani A. Amorim

Additional information is available at the end of the chapter

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Abstract

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

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

1. Introduction

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

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

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

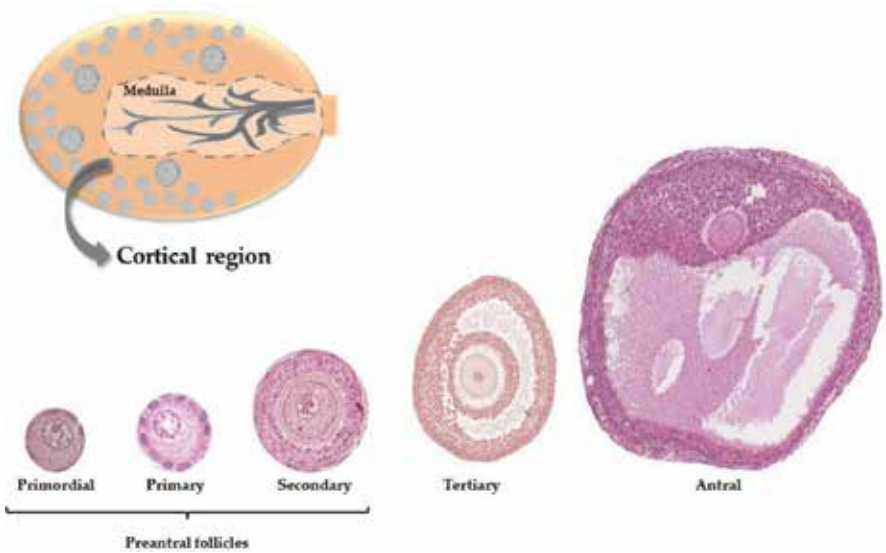

Figure 1. Ovary organization and follicle classification according to developmental stage. Female germ cells are enclosed in follicles that are localized in the cortical region of the ovary, the external layers. The inner layer contains mainly blood vessels and ligaments and is termed the medulla. Once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

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

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

2. Why should we cryopreserve ovarian preantral follicles?

2.1. Indications in women

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

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

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

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

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

2.2. Indications in other mammalian species

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

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

3. Methods for preantral follicle cryopreservation

3.1. Determining the cryopreservation protocol

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

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

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

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

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

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

3.2. Ovarian tissue cryopreservation

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

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

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

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

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

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

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

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

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

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

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

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

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

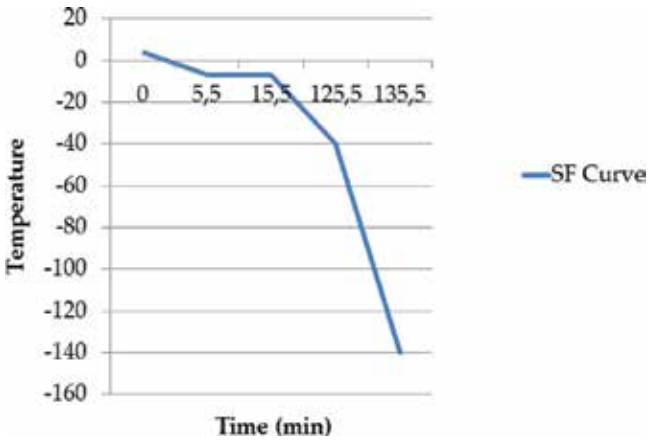

Figure 2. A schematic demonstration of the cooling rate applied by Gosden *et al.* [4] for slow-freezing of sheep ovarian tissue. Usually, cooling rates are performed in an automatic freezer, useful for cooling samples up to −140°C. SL, slow-freezing.

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

3.3. Isolated follicles cryopreservation

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

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

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

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

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

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

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

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

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

4. Warming rate and CPA removal

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

5. Conclusion and final considerations

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

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

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

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

CPA, cryoprotective agent.

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

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

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Author details

Ellen C.R. Leonel[1,2], Carolina M. Lucci[3] and Christiani A. Amorim[2]*

*Address all correspondence to: christiani.amorim@uclouvain.be

1 Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (UNESP), São José do Rio Preto, Brazil

2 Laboratory of Gynecology, Institute of Experimental and Clinical Research, Université Catholique de Louvain (UCL), Brussels, Belgium

3 Department of Physiology, Institute of Biological Sciences, University of Brasília (UnB), Brasília, Brazil

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Chapter 6

Vitrification: Fundamental Principles and Its Application for Cryopreservation of Human Reproductive Cells

Mitchel C. Schiewe and Steven F. Mullen

Additional information is available at the end of the chapter

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Abstract

The fundamental understanding of cryobiology through experimentation in the 1960s, 1970s, and 1980s has led to the development of today's vitrification technology. Although human embryo and oocyte vitrification was slow to evolve, it has become an invaluable technology in the field of reproductive medicine. The aim of this chapter is to discuss some of the underlying basic principles behind forming a metastable glass phase during rapid cooling in liquid nitrogen (LN_2) and the prevention of recrystallization events upon warming. We then highlight how this understanding has led to its highly effective and reliable usage in clinical IVF. Furthermore, we describe how quality control factors (e.g., ease of use, repeatability, reliability, labeling security, and cryostorage safety) can vary between vitrification device systems, potentially influencing clinical outcomes and creating possible liability issues. An open-minded approach to continued experimentation is a necessity, especially pertaining to oocyte freeze preservation, if we are to optimize the vitrification of reproductive cells and tissue in the future.

Keywords: cryopreservation, embryo, oocyte, quality control, vitrification

1. Introduction

Studies investigating the effect of cooling on biological cells have been conducted since at least the late 1700s [1]. For much of this history, relatively uncontrolled methods were utilized. The discovery [2], or rediscovery [3], of the protective effects of small-molecule solutes such as glycerol and sucrose, when incorporated into carrier solutions used during cooling and warming, has greatly expanded the efforts to develop robust freeze preservation methods.

An exponential number of reports successfully preserving biological material have been published in the scientific literature over the last six decades.

Because long-term preservation of biomaterial requires cessation of biochemical reactions, storing this material is typically done at very low subzero temperatures. As a result, ice usually forms in the sample, often with deleterious consequences. The avoidance of ice formation, particularly intracellular ice, is known to be one of the most important factors contributing to successful cryopreservation [4]. Preventing intracellular ice formation (IIF) during cooling and warming, and maintaining cellular viability during this process, is achieved by increasing the concentration of solutes in the cytoplasm. This is done by one of the two ways. The first way, referred to as either slow cooling or equilibrium freezing, allows the cell's sufficient time to dehydrate as a result of extracellular ice formation and subsequent exosmosis during the cooling process. This dehydration increases the concentration of the solutes in the cytoplasm, preventing lethal intracellular ice formation during subsequent cooling and warming if done appropriately. A thorough discussion of the principles of cryopreservation has been reviewed by Peter Mazur [5]. The second method intentionally loads high concentrations of solutes into cells prior to cooling below the freezing point of the solution. With this method, the solution containing the cells (and the cells themselves) maintains an amorphous state during subsequent cooling and warming. This later procedure, generally referred to as vitrification, is the focus of the current chapter.

While practical methods to successfully vitrify cells were not developed until the mid-1980s, the concept of vitrification as a means for cryopreservation has a much longer history. In the 1930s, Stiles suggested that, with the use of very rapid cooling, cytoplasm may not extensively crystallize. The result of which could be maintenance of the system [6]. In 1937, Father Basile J. Luyet, one of the founding members of the Society for Cryobiology and its first President, developed the concept of vitrification into a major research proposal [7]. For reasons described elsewhere [8], Father Luyet never succeeded in developing a method to vitrify cells successfully but laid the foundation for the development of vitrification methods by those that followed.

It was not until the 1980s that a method for reviving cells after vitrification was demonstrated unequivocally [9]. Gregory Fahy had worked on developing methods for vitrification for over a decade, and his contributions, coupled with those of William Rall, proved that mouse embryos could be vitrified using several methods. It was subsequently shown that a practical, ambient approach to vitrify and warm embryos could efficiently and effectively produce healthy live births in the mouse [10] and sheep model [11].

After the initial report in 1985, numerous experiments describing successful vitrification of embryos from other species were published. It is beyond the scope of the present work to describe these reports in detail, but such information can be obtained in recently published reviews [12–16].

2. Achieving a vitreous state when cooling an aqueous solution: physical aspects

At temperatures above an aqueous solution's melting point, the water remains liquid as a result of the Gibbs free energy being lower in comparison to that in the solid phase [17]. As

a solution is cooled, it becomes progressively more favorable for ice to form. However, even when the temperature goes below the thermodynamic equilibrium point, ice formation is initially unfavorable as a result of an energy barrier to ice nucleation.

As cooling proceeds, it becomes more favorable for ice nuclei to form. This is often the result of water molecules becoming arranged in a favorable configuration on a foreign particle suspended in the solution. This process is called heterogeneous nucleation. Homogeneous nucleation (i.e., where an ice nuclei forms as a result of self-aggregation of water molecules) is not favorable until relatively low temperatures (~−39°C in pure water). Therefore, preventing heterogeneous nucleation during cooling is important to attaining the vitreous state.

Avoiding homogeneous nucleation is difficult when cooling dilute solutions. However, increasing the concentration of solutes in a solution depresses the homogeneous nucleation temperature (T_{hom}), and it is possible to depress T_{hom} below the glass transition temperature (T_g) with a sufficient solute concentration [18]. Unfortunately, solutions with solute concentrations high enough to depress T_{hom} below T_g are usually too toxic to biological systems to be of practical use.

Fortunately, it is possible to depress T_{hom} low enough with relatively nontoxic solutions such that kinetics begins to exert an appreciable effect on the probability of ice nucleation and growth. It is the combination of thermodynamic and kinetic effects that allow ice crystal nucleation and growth to be avoided during cooling of these solutions and is the means by which vitrification of oocytes and embryos are achieved presently.

During warming, the likelihood that extensive ice crystal formation will occur is greater for a given solution having been cooled at a specific rate, resulting in markedly higher warming rates being necessary to maintain the vitreous state. The mechanism behind devitrification events has been previously discussed [19]. In brief, during cooling, the nucleation of ice in a solution can be prevented until very low temperatures. These ice nuclei are often very small (submicroscopic), and a solution cooled with only moderate concentrations of cryoprotectants is, for all intent and purposes, vitreous. However, these nuclei remain present during warming up to the melting point of the solution. Because crystal growth occurs more rapidly as the temperature increases, if warming is not extremely rapid (greater than the cooling rate), extensive ice crystal growth will occur from these previously formed nuclei. This phenomenon is referred to in the literature as devitrification and is believed to be just as damaging to biomaterial as ice crystal formation during cooling [19]. Hence, developing cryopreservation methods to avoid extensive crystallization rely upon rapid warming more so than rapid cooling, a point often overlooked in the literature (See **Figure 1**).

It should be pointed out that ice formation during warming is not necessarily damaging to biological systems, particularly if it occurs for only very brief periods of time and the crystals remain very small. It is believed that this may be due, in part, to the type of ice crystal structure initially formed at low temperatures, as ice has many crystalline forms. However, if the system is afforded sufficient time during warming, the molecules in the ice crystals may rearrange to form the more favorable (i.e., from a thermodynamic standpoint) hexagonal crystal structure as well as larger crystals. It is believed that this structure of ice is the most damaging to biological systems [19].

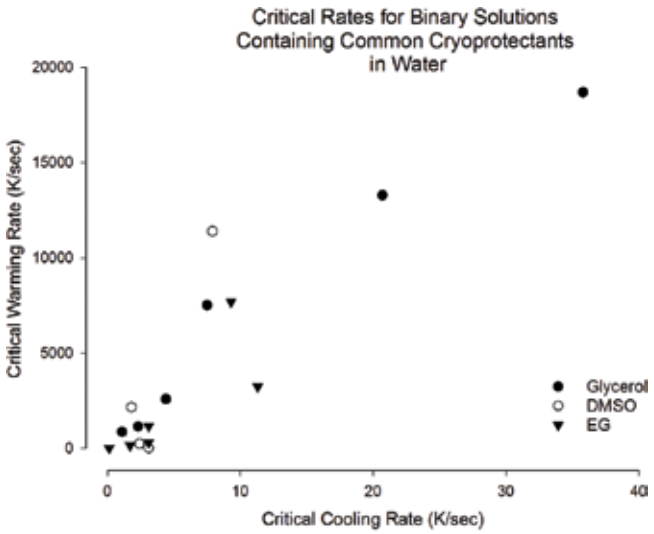

Figure 1. The relationship between the critical cooling rate (CCR) and the critical warming rate (CWR) of solutions containing a permeating cryoprotectant dissolved in water is shown. Note that the CWR is generally two to three orders of magnitude greater than the CCR (Data from Table 1 in Hopkins et al. 2012 [21]).

Historically, studies investigating the relationships between devitrification and rates of temperature change as well as solute concentrations have been limited to relatively slow cooling and warming rates due to technical limitations (<100 K/min). As such, estimations of the critical concentrations for the realm of cooling and warming rates that are utilized in the embryology laboratory have often been extrapolated from those data [20] with uncertain accuracy. Fortunately, a recent study has shed light on the critical concentration of solutes in the ranges of cooling and warming rates encountered in oocyte and embryo vitrification [21]. Some of the results from that study were not surprising—revealing a greater critical warming rate compared to the cooling rate for a given solute concentration (**Figure 1**), for example. Also, the critical warming rate is strongly dependent upon cooling rate, even at the high rates of warming in the latter study. Other results, however, were enlightening. The experimental data suggests that the critical warming rate is in fact lower than previously estimated from theoretical models (c.f. [22], as one example) suggesting that the current systems may be more stable than previously estimated. On the contrary, experimental ice growth after nucleation was much faster than theoretically predicted [23], approaching 25 μm sec^{-1} at −33°C from approximately zero growth below −80°C. From a practical standpoint, this means that ice, starting at a nucleus in the center of a mature human oocyte (radius = 63 μm; [20]), would proceed to the edge of the cell at −30°C in roughly 2 seconds. This highlights the importance of warming as quickly as possible when vitrifying oocytes and embryos.

3. Cryoprotectants as components of vitrification solutions

An exhaustive review of this topic is beyond the scope of the current work, and, therefore, interested readers are referred to thoughtful reviews published previously, as well as the

primary literature cited within those papers [24–27]. As with freezing methods, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and glycerol are common components of vitrification solutions used for reproductive cells and tissues. These solutes possess favorable properties such as high solubility and cellular permeability and relatively low toxicity. For systems where extremely rapid cooling and warming are not possible (i.e., pieces of tissue and the entire organs), these compounds must be used in fairly high concentrations to preclude ice formation. Unfortunately, at these concentrations, the toxicity of the solutions becomes a serious concern, and investigations to determine superior vitrification solutes have been conducted as a result [27, 28].

In some investigations the search for superior vitrificants has been conducted by assessing the effect on the concentration necessary to vitrify (CNV) of the molecular structure of the closely related compounds. As an example, the position of the hydroxyl side groups (and the associated presence or absence of methyl groups) on diols has a significant effect on the vitrifiability of solutions. For example, at low cooling rates, the concentration of 1,2-propanediol necessary for vitrification is 44% by mass, whereas with the isomeric form 1,3-propanediol, the CNV is increased substantially (to 57% by mass), making the more commonly used isomer a superior vitrificant. Similarly, for a fixed concentration of solute, the position of the hydroxyl pairs plays a significant role in attaining and maintaining an amorphous state. A solution of 30% 1,3-butanediol (by mass) dissolved in phosphate-buffered saline containing 4% sorbitol has a critical warming rate (i.e., rate needed to prevent adverse devitrification effects) of 2.73×10^{9}°C/min. However, this rate is reduced by nearly four orders of magnitude for its isomer 2,3-butanediol (2.9×10^{5}°C/min) [29].

Similarly, adding methyl groups to commonly used diols greatly enhances their stability [30]. The critical warming rates for 50% (by mass) ethylene glycol, ethylene glycol monomethyl ether, and ethylene glycol dimethyl ether are 250, 80, and 5°C/min, respectively. About 30% propylene glycol monomethyl ether has a critical warming rate of 7×10^{3}°C/min, whereas 35% propylene glycol has a critical warming rate of 2×10^{7}°C/min [30]. These effects are believed to be strongly associated with the relative ability of the compounds to hydrogen bond with water [27].

Unfortunately, a significant correlation between vitrifiability and biological toxicity has been noted [31], making what might seem to be relatively simple modifications to currently used vitrification solutions too toxic for practical applications. One alternative has been the inclusion of nonpermeating solutes as a means to reduce the concentration of permeating (and presumably more toxic) compounds in vitrification solutions. There are two general classes of agents used, relatively small sugars (usually disaccharides like sucrose and trehalose) and larger molecular weight polymers (i.e., Ficoll and polyvinyl alcohol). The former can enhance the vitrifiability of solutions for a given concentration of permeating agent(s) and also enhance the vitrifiability of cytoplasm via dehydration. The latter generally have lower osmotic effects on cells but can enhance vitrifiability by one or more means [18].

Due to their large size, these macromolecules contribute markedly to the viscosity of vitrification solutions, thus suppressing the kinetics of molecular motion and consequent ice forming tendency as solutions are cooled. Additional developments include the discovery that certain polymers are able to interfere with ice nucleation and growth, presumably by directly

interacting with the surface of ice nuclei and small crystals [32]. These types of compounds (e.g., X-1000 and Z-1000 as marketed by 21st Medicine, Inc.) [32, 33] are becoming common additives to vitrification solutions as they are particularly effective in this regard. It should be pointed out that the ice blockers may not make a significant difference for vitrification under so-called ultrarapid cooling regimes. However, there are benefits to vitrifying by cooling and warming more slowly [34, 35], and such methods may eventually prove to be superior to some of those currently being utilized.

4. Quality control considerations in vitrification systems

Since the first effective method for vitrification was demonstrated using mouse embryos [9], results from numerous experiments designed to vitrify embryos from other mammals have been reported. Investigations at that early time period utilized standard 0.25 ml cryostraws as sample carriers [10, 36]. Chilling injury was determined to be a challenge to cryopreserving bovine and porcine embryos, particularly cleavage-stage embryos. This was also discovered to be a serious concern for oocytes. Following the elucidation of the kinetics of chilling injury on cattle oocytes [37], more successful methods to cryopreserve these sensitive cells developed as a result of increasing the rate of cooling. This was achieved by using approaches that reduced the volume of solution being cooled and reducing or eliminating the effects of the sample carriers on heat transfer from the sample [37].

In the adoption of vitrification to the human ART industry, thinner straws and flat to semi-flat sample supports composed of various materials gained prominence for use to increase the cooling and warming rates of the samples [38]. While these devices tended to improve outcomes, many have relied upon directly exposing the samples to liquid nitrogen [39]. This results in an increased theoretical risk of sample cross-contamination from contaminated liquid nitrogen [40], yet no such disease transmission has ever occurred via an embryo or oocyte [41]. Furthermore, these systems have been reported to be very challenging to use, resulting in a significant "technical signature" of the outcomes [42–44]. Commercial influences have pushed vitrification devices into the marketplace, in fact more than 25 different device systems have been utilized. This commercial push to market devices has created serious potential quality control (QC) problems, such as inherent design flaws of some devices in secure labeling, open system storage, and suboptimal recovery and inconsistent survival rates; these factors could present unnecessary and undesirable industry variation with substandard outcomes. While a device system may be perfected, or not, within a laboratory, when samples are transferred for warming to another laboratory, reduced outcomes may occur. This interlaboratory variation poses potential liability issues to both programs [45, 46]. Even when both programs are competent in their procedures, the relationships between cooling and warming thermodynamics as discussed above can have applied consequences. For example, open device systems like Cryo-Locks, Cryo-Tops, or Cryo-Tech that have become a worldwide industry standard emphasize the use of micro-volumes of DMSO/EG solutions varying from 0.5 to 0.05 μl. This 10-fold variation in volume, or greater, directly influences both the risk of dehydration and cooling/warming rate potentials of the sample. A program

emphasizing ultralow volumes utilizing visual dehydration (i.e., complete loss of solution seen around sample) prior to LN_2 direct exposure to maximize ultrarapid cooling rates is likely exposing oocytes/embryos to potentially damaging or unnecessary osmotic stress and an increased risk of suboptimal warming rates by an end user unfamiliar with their particular vitrification nuance in the technique. In turn, a poor outcome by the end user is not necessarily a reflection of their competence. Device systems using higher volumes, or more importantly set volumes, are less vulnerable to warming rate variation under standard conditions.

Alternative efforts have focused on developing aseptic vitrification devices/procedures that offer simplicity and reliability of use, high survival/viability rates, and biosecurity. The CBS™ 0.3 ml embryo/semen straw is an ideal storage container, offering biosecurity, and tamper-proof, dual-colored labeling for ease of identification. In conjunction with vitrifying in an open pulled straw (OPS; [47]), a cut standard straw (CSS; [48]), or sterile flexipette (microSecure-VTF; [44]), effective low-cost options are proving to be very practical and successful, in contrast to expensive commercial systems like the CBS™-HSV and VitriSafe™ devices [49, 50] . The success of implementing these double-container systems is predicated more on achieving rapid warming rates (in excess of 5000°C/min) than on their moderately rapid cooling rates (up to 1500°C/min). Thus, the simplicity of the system and the ability to effectively remove the device for rapid warming are critical factors to these aseptic methods. These factors were taken into consideration in the development and validation of the microSecure-VTF system [51], which has proven to be a user-friendly technique offering high inter-technician repeatability and reliability (100% recovery rates), high survival rates, and high live birth rates with human oocytes and blastocysts [44, 52, 53]. There are also hybrid vitrification device systems like Rapid-i [54] and the Cryotop SC [55] which ultrarapidly cool the device prior to sealing them into a straw container under LN_2 vapor conditions, placing the container at risk of incomplete seals (i.e., particularly the Cryotop SC and homemade cut straw-double container systems). The latter event could allow LN_2 seepage to occur and problematic warming events to transpire if not accounted for properly [56]. Variations in device systems that place the end user at risk of unexpected poor outcomes, like non-recovery and high degeneration rates, create serious liability issues to IVF programs, as recently discussed [46]. Liability concerns can also stem from poor manufacturer design or user compliance to quality management practices.

When contemplating which vitrification device to use, there are some critical factors to evaluate to accurately judge its potential usefulness [57]. We believe it is important to assess labeling potential, technical ease, simplicity and repeatability, LN_2 storage, recovery potential, and survivability. A device that offers secure (internalized), dual-colorized labeled containers (e.g., CBS™ embryo straws) is considered optimum. In evaluating technical ease, one should assess handling simplicity/repeatability of cryo-loading and warming, as well as identification potential. Can the desired vitrified sample be promptly, clearly, and accurately identified without ambient exposure? In terms of LN_2 exposure, is the device closed with secure seals, easy to handle, well protected, and space efficient (e.g., greater than five devices/goblet)? Furthermore, technician's safety in the handling of devices in LN_2 should be seriously evaluated and not overlooked. Finally, one should determine the recovery and survival potential of using a particular device before implementing it into commercial practice. In terms of

"technical signature," remember that any good laboratory can master a device with sufficient practice and experience, but can an inexperienced lab using that same device have comparable results? Therein lies the rub, a potential QC nightmare awaits as so many different types of suboptimum vitrification devices have been mass marketed without full consideration to the factors listed above.

Ultimately, the successful application of all vitrification methods, independent of the device used, is dependent on three principle quality control components (i.e., the 3 "Cs" to successful vitrification): clarity of the mind (i.e., organization), concentration (i.e., focus on task at hand), and consistency (meticulous, technical repeatability; [44]). For example, complete organization (i.e., clarity) is imperative to avoid any variation in strictly timed dilution and loading/plunging/warming steps. Meanwhile, the cryo-dish setup and routine manner of warming and diluting oocytes and embryos (intra- and inter-solution steps, i.e., consistency) can be critical to the effective and timely use and reuse of vitrification solutions. Because of the rate-limiting nature of these dilution steps to avoid toxicity, user concentration and focus are important to insure that multiple straws and devices can be prepared in a short, precise time period. It is the latter issue that poses the greatest challenges to animal industry application of vitrification where large numbers of oocytes and blastocysts may need to be processed in a given day (e.g., in vitro production facility).

5. Experimental aspects of today's clinical vitrification

After 20 years of development, vitrification has transformed the IVF industry, with regard to oocyte cryobanking [58–60] and the justified adoption of vitrification-all IVF cycles [61] in conjunction with blastocyst culture and micromanipulation. The combined use of nontoxic levels of permeating cryoprotective agents, coupled with supplemental macromolecules, as discussed above, has facilitated the safe vitrification of human oocytes and embryos. Today, blastocysts are vitrified with great confidence that their fresh-state viability will be completely sustained. This is particularly true in conjunction with blastocyst biopsy/PGS-single embryo transfer application ([62, 63] where over 99% survival can be typically achieved [64] along with efficient pregnancy success across all age groups following single euploid embryo transfer [65]. Embryo and oocyte vitrification has been the most significant procedure applied to the ART industry since the development of ICSI [44]. In our own experimental efforts to verify the safety and reliability of μS-VTF in a metastable solution (>7.9 M glycerol/DMSO-free; Innovative Cryo Enterprises, NJ, USA), we determined that blastocyst viability was sustained after up to five times re-vitrified (rVTF) with and without equilibrating sucrose dilutions post-warming [66]. Although the commonly used 15% DMSO/15% EG commercial vitrification solution is less cytotoxic to human blastocysts following extended exposure (>10 min; **Figure 2**), its inferior metastability is less resilient to repeated rVTF.

In the last decade, oocyte vitrification has proven to be a reliable option for the "fertility preservation" of women facing potential sterilization medical treatments. Based on the normal health and well-being of live-born babies, and consistently good survival, fertilization,

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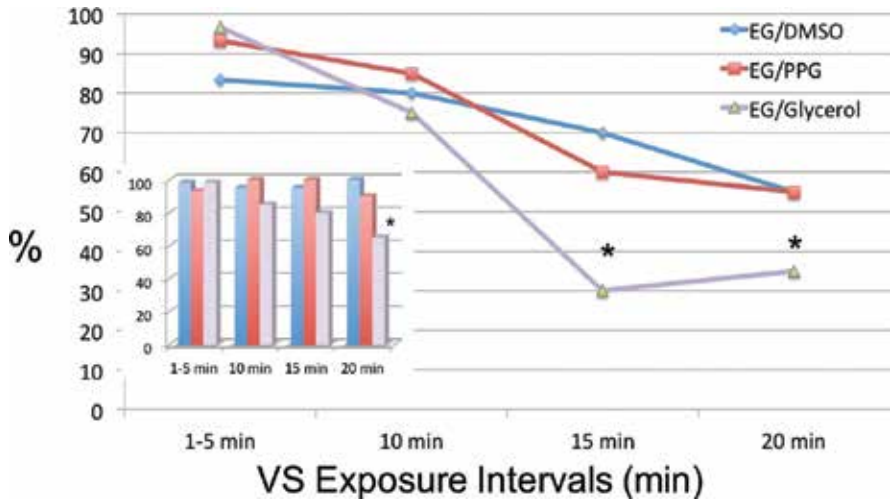

Figure 2. The effect of vitrification solution (VS) type by exposure interval is conveyed in terms of initial post-warming survival (0 hr; inner bar graph) and 24 h sustained development (line graph). Statistical differences ($p < 0.05$) between groups within interval are indicated by an asterisk (*). The more concentrated, metastable EG-glycerol solution tended ($p > 0.10$) to show reduced survival and development of human blastocysts after 10 min of exposure, with overt differences in toxicity seen by 15 min.

and pregnancy rates in some randomized clinical trials, oocyte cryopreservation technology was deemed "nonexperimental" in 2013 by the American Society for Reproductive Medicine (ASRM; [67]). It should be noted that the determining factor to remove the "experimental" classification was dictated by medical insurance factors to aid the former female population. Although oocyte vitrification has numerous potential clinical benefits, including IVF cycle rescue and elective fertility preservation when a sperm provider fails to produce or is unavailable, respectively, its "nonexperimental" classification has been potentially misleading [46]. Blastocysts derived from vitrified oocytes are comparable to those derived from fresh oocytes, in terms of euploidy and live birth rate potential [68, 69]; however, the overall developmental competence of zygotes to the blastocyst stage continues to be delayed and reduced overall [69].

Little to no progress has been made over the past 5 years to correct or understand why overall blastocyst development may be reduced between cryopreserved batches. The inefficiencies of oocyte cryopreservation go beyond the device system used or technical variation and undoubtedly rest on improving our understanding of the membrane integrity and cytoplasmic sensitivity of this large single cell [45, 70, 71]. There remains a need to understand more about cytosolic factors at the level of gene regulation and energetics of vitrified-warmed oocytes that could be responsible for decreasing their developmental potential [72–74]. Due to the high costs, resource availability, and ethical considerations of generating human oocytes for experimentation, research progress is slow but necessary. There is a need to continue exploring the role of safer, metastable vitrification practices (e.g., solutions, equilibration intervals, dilution methods), as well as the cytoplasmic preparedness of oocytes to be cryopreserved.

6. Summary

As with all ART procedures, there is always room for improvement in their application and outcomes. Steady advancements in reproductive tissue and oocyte vitrification will likely require continued experimentation to further understand membrane biomechanics and the role of extracellular stabilizing additives (e.g., hyaluronate, hydrocellulose, and butylated hydroxytoluene) and ice blocking agents (e.g., polyvinyl alcohol polymer), organelle functionality and gene expression, cryoprotectant interactions, and possible toxicities. Furthermore, quality management improvements aimed to reduce technical variation will all prove critical to optimizing vitrification in the future. Ideally, vitrification systems require mindfulness to quality control issues to enhance procedural consistency and repeatability. Our common goal should be to eliminate technical signature by reducing intra- and interlaboratory variation. Indeed, our future ability to sustain cellular viability and physiological processes is infinite in the wondrous world of glass formation and the controlled elimination of recrystallization events.

Author details

Mitchel C. Schiewe[1]* and Steven F. Mullen[2]

*Address all correspondence to: mschiewe@ovationfertility.com

1 Ovation Fertility, Newport Beach, CA, USA

2 Cook Regentec, Bloomington, IN, USA

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Section 3

Application of Cryopreservation in Veterinary Medicine Researches

Chapter 7

Biological Signals of Sperm Membrane Resistance to Cryoinjury in Boars

Julian Valencia and Francisco Javier Henao

Additional information is available at the end of the chapter

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Abstract

Despite the great progress achieved in the cryopreservation of boar semen, it has not been possible to effectively counteract the negative effects on fertility and prolificacy. The boar sperm membrane (SpM) has a particular composition of phospholipids, cholesterol, and proteins that make it highly sensitive to freezing. Just at the beginning and during the freezing protocol, the sperm are exposed to factors that destabilize the membrane and increase the sensitivity to cholesterol efflux and lipid peroxidation. This is a series of events similar to physiological capacitation; they are commonly called cryocapacitation. All the molecules reported as freezability marker and those considered potential markers are directly or indirectly related to the physiology of the SpM. The above gives rise to intensify studies tending to assess their importance as facilitators of the boar semen freezing.

Keywords: boars, reproduction, boar, semen freezability, biological signals

1. Introduction

The cryopreservation of boar semen is unquestionably an indispensable technology in the development of actions for the conformation of germplasm banks and commercial genetic improvement plans. Despite the great progress achieved in different topics related to this problem, it has not been possible to effectively counteract the damage caused by the freezing protocols to the sperm cell with the usual negative effects on fertility and prolificacy. The current extensive knowledge about the physiological, morphological, and molecular characteristics has been pointed out about many of the peculiarities of the (SpM) of the boar. Each day there is greater clarity about the lipid composition and its dynamics in the fluid mosaic and about the protein

fraction and its participation in crucial events that guarantee the integrity of the sperm and the complete fulfillment of its reproductive function. The present chapter is oriented to confront from the basic morphology and physiology of the cell and especially the membrane, the damages caused by the cryopreservation technique with the evidences registered around possible resistance phenomena characterized by abundance, and lack or absence of certain molecules from both the spermatozoa and the seminal plasma (SP). The aforementioned confrontation has focused on studies that classify boars according to the freezability of their semen, by virtue of the behavior of molecules, especially proteins, currently nominated as freezability markers.

2. Sperm membrane characteristics of boar

2.1. Lipids

The cell membrane is a highly fluid and dynamic lipid bilayer, composed mainly of phospholipids, cholesterol, and proteins. Thanks to the amphipathic character of phospholipids and cholesterol, the cellular membrane functions as a barrier between intracellular and extracellular environments [1]. The phospholipids have a hydrophilic head or polar group and two hydrophobic hydrocarbon acyl chains [2]. The polar group is constituted for a phosphate esterified with glycerol (short-chain alcohol) in phosphoglycerolipids (PG) or sphingosine (long-chain alcohol) in sphingomyelin (SM) [3, 4]. Likewise, cholesterol structure consists of a hydrophilic hydroxyl group linked to a hydrophobic steroid ring structure of cyclopentanoperhydrophenantrene with a hydrocarbon tail [3]. In the phosphate group of the PG, a second alcohol or an amino alcohol can also be attached and different phospholipids are produced: phosphatidylinositol (PI) (alcohol inositol), phosphatidylserine (PS) (aminoal cohol serine), phosphatidylethanolamine (PE) (amino alcohol ethanolamine), and phosphatidylcholine (PC) (amino alcohol choline) [3, 5]. PG are in greater abundance than SM [2], and in boar SpM, PC and PE are found in greater quantities than PS and PI [6]. In boar SpM, the outer leaflet is mainly composed of PC and SM and the inner leaflet by PS and PE, possibly by the action of an aminophospholipid translocase [4, 7]. This translocase is ATP dependent and causes a rapid movement of PS and PE toward the inner leaflet of the membrane [7, 8].

In human, ram, rabbit, bull, and boar spermatozoa, the SpM has higher amount of long-chain polyunsaturated fatty acids (PUFAs) with cis configuration than the membranes of the somatic cells [9–11]. In addition, differences have been found in the proportion of unsaturated to saturated [12].

The most abundant fatty acids in the phospholipids of the boar SpM are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, n-9), and the PUFAs: docosapentaenoic acid (DPA; 22:5, n-6) and docosahexaenoic acid (DHA; 22:6, n-3) [10].

The sterols are the second most abundant class of lipids in the SpM, mainly cholesterol (24% from total lipids) and in a lesser proportion, desmosterol [6, 13–17]. The ratio of cholesterol to phospholipid is 0.26 compared with 0.45 in the bull, 0.3 in the rooster, and 0.36 in the stallion [6]. In addition, cholesterol is distributed asymmetrically in the lipid bilayer, with greater amount

in the outer leaflet [15], by its affinity with PC and SM [18]. The cholesterol molecules are inserted between the phospholipids and interact with the fatty acids, and their rigid steroid structure provides stability and organization to the membrane [2, 19].

2.2. Fluidity and selective permeability of the boar SpM

The fluidity of the boar SpM depends on the temperature [20], the hydrocarbon acyl chains of the phospholipids [17, 21], the sterol content [19], and the charge of the polar groups of the phospholipids [22]. High amount of PUFAs with cis configuration in the boar SpM affects the degree of compaction of the phospholipids and increases the membrane fluidity [2, 9, 11, 22].

Depending on the temperature, the lipids of the membrane can be in liquid crystalline phase or in gel phase [22, 23]. The temperature, at which the lipids pass from one phase to another, is the phase transition temperature (Tm). Above Tm, the membrane is liquid, and the cholesterol limits the lateral diffusion of phospholipids and proteins, so that it maintains the membrane stability and moderates the fluidity. Below Tm, the membrane is in a gel phase, the cholesterol increasing the membrane fluidity [2, 25] and maintaining its stabilizing function [19, 23].

As the boar SpM is composed of different types of lipids (saturated fatty acids, PUFA, cholesterol, desmosterol, and others) [9, 16], the phase transition occurs in a temperature range between 30 and 5°C [23], which leads to lipid phase separations and irreversible alterations, when it is exposed to low temperatures [15, 23, 24].

The property of selective permeability of the membrane is determined by the presence of protein ionic channels and specific protein transporters [2]. The boar SpM has a ratio of phospholipids to proteins of approx. 0.68 [14], and it has different membrane proteins involved in capacitation, acrosome reaction, motility, and cell volume regulation, such as: HCO_3^-/Cl^- exchanger [4], voltage-dependent anion channel 2 (VDAC2) [26, 27], calmodulin-sensitive Ca^{2+}-ATPase [28], ATP-binding cassette transporters, class B scavenger receptor [19], K^+ and Cl^- channels [29] and aquaporins (AQP) [30].

2.3. Domains and microdomains in boar SpM

The boar spermatozoon has a morphology according to its physiology [31]. Each of its parts is highly differentiated by polarized membrane domains [32]. Protein structures separate these domains, the posterior ring separates the membrane head from the midpiece membrane, and the annular ring separates the midpiece membrane from the flagellum membrane [4]. The membrane of the boar sperm head can be subdivided into four regions: apical, acrosomal, equatorial segment, and post-acrosomal [31, 33, 34]. These regions are highly heterogeneous [32], in which it is evidenced by a series of specific sperm glycolipids, called seminolipids, in the outer leaflet of lipid bilayer, in the apical region of recently ejaculated spermatozoa from the boar [35, 36].

The boar SpM in liquid crystalline phase is in lipid-disordered membrane phase, with high lateral diffusion of lipids and proteins due to low amount of cholesterol [19, 37]. The outer leaflet of somatic cells membrane has lipid-ordered microdomains known as lipid rafts that are rich in SM, glycosphingolipids, saturated phospholipids, and proteins, ordered by the

presence of high amount of cholesterol [2, 38, 39]; they function as platforms for molecular signaling, cell adhesion, and cell-to-cell interaction [38, 39]. Lipid rafts have been identified in both boar spermatozoa and sow oocytes [40], and are associated with maturation, capacitation, acrosome reaction, and gamete interaction [37, 40, 41].

3. Physiological events in the SpM between ejaculation and fecundation

3.1. Characteristic events occurred during ejaculation

During boar ejaculation, mature spermatozoa from the epididymis tail are mixed with the secretions of the accessory sex glands [33, 42]; in this moment occur different events: first, the change of osmolarity from 331 mOsm/L in the epididymis tail to 300 mOsm/L of the SP [43–46] produces swelling of the sperm and the activation of a regulatory volume (RV) process (regulatory volume decrease (RVD) in this case) [46, 47]; second, the elimination of cytoplasmic droplet by increasing the levels of fructose [48] with possible participation of RVD [45, 49]; and third, the union of spermadhesins: AQN-1, AQN-2, AQN-3, AWN-1, AWN-2, DQH, PSP I, and PSP II (among other proteins) from the SP to the SpM, as decapacitating factors [50–52].

3.2. Regulation of cellular volume (CV)

The maintenance of CV and the adequate concentration of ions and molecules are vital for normal sperm physiology [53]. CV is determinate by the relationship between the intracellular content and the osmolarity of the extracellular medium [54]. Drevius [55] states that spermatozoa behave like perfect osmometers. The sperm exposure to anisotonic conditions causes a cellular swelling or shrinkage, phenomena counteracted by the activation of a regulatory volume process [46]. This process can be RVD or regulatory volume increase (RVI) depending on the osmotic change [29, 46] which lead to influx or efflux of water and osmolytes and ions [56]. During the epididymal maturation, the sperm acquires the ability to regulate cell volume [56], in the case of the boar, the sperm undergo an osmotic change that goes from approximately 296 mOsm/L in the rete testis until reaching around 331 mOsm/L in the tail of the epididymis [43]. Then, at ejaculation, sperm are subjected to an osmotic gradient, from a hyperosmotic environment in the tail of the epididymis to the isoosmotic conditions of the PS (around 300 mOsm/L) [43, 44]. Because of this osmotic change, there is influx of water into the sperm to reestablish the osmotic equilibrium through the dilution of the intracellular osmotic content and cell swelling occurs [57]. Sperm swelling induced by hypoosmosis produces the opening of K^+ ion channels, allowing the exit of intracellular K^+ ($[K^+]i$) under a concentration gradient [29, 46, 56]. In parallel, Cl^- ion channels are opened to promote the exit of Cl^- intracellular ($[Cl^-]i$) in order to achieve an electrical balance [29, 46]. The exit of the $[K^+]i$ and $[Cl^-]i$ reduces intracellular osmolarity, water loss, and reduction of cell volume [29, 53].

RVD's pathways are mediated by the enzymes protein kinase C (PKC) and protein phosphatase 1 (PP1), which change the balance of phosphorylation-dephosphorylation of threonine and serine residues [53, 58–60]. Using the boar spermatozoa as a model, Petrunkina [58] found that the

phosphorylation activity of PKC seems to be related with deactivation of RVD through closing and keeping closed the ion channels, especially Cl^- ion channel, while the dephosphorylation activity of PP1 produces the opposite effect. These authors found that by activating phosphodiesterase that reduces cAMP levels, it results in inactivation of RVD, and by stimulating adenylate cyclase (AC) in order to increase cAMP levels, under hypoosmotic conditions, it is activated RVD (opening of ionic channels). However, in isosmotic conditions, high levels of cAMP increase the cell volume by premature activation of RVD-related channels, which consequently produces the entry of Cl^- and Na^+ under a concentration gradient, increasing intracellular osmolarity. This increase in cell volume under isoosmotic conditions also occurred with the inhibition of PKC.

3.3. Main events occurred between sperm deposition in the cervix and its arrival to the spermatic reservoir (SR)

Between the cervix and the SR, sperm must overcome barrier represented by the cervical mucus and the polymorphonuclear neutrophils and T cells; the presence of semen in the uterine lumen causes endometrial inflammation and recruitment of polymorphonuclear neutrophils and T cells that attack the spermatozoa [61] and an adequate volume regulation have relation with migration capacity through the cervical mucus [62]. In the SR spermatozoa are protected from polymorphonuclear neutrophils, thanks to the bind of spermadhesin AQN-1 of the SpM with lysosome-associated membrane protein (LAMP) receptors 1 and 2 of the membrane oviductal epithelium [42, 63–65]. There is an association of good sperm RV capacity with high farrowing rates in pigs [54]. Boar sperm with problems in RV capacity [41], as well as with morphological alterations or cytoplasmic drops, and epididymal sperm have a negative relationship with binding index to pig oviductal epithelium [66]. Likewise, spermatozoa with chromatin instability, which have a high relationship with the retention of cytoplasmic droplets and with immatures, have low binding capacity to the oviduct epithelium [67]. A study developed by [68] showed that proteins from the plasma membrane of the oviduct epithelium suppress the activation of bicarbonate-linked motility selectively.

3.4. Relationship between boar sperm capacitation and CV regulation

During sperm capacitation there are changes in membrane permeability and ion entry and exit [69], and sperm undergoes osmotic changes in the sow's reproductive tract [29, 47, 62]; thus, it is necessary to establish a molecular model of capacitation that involves the cellular RV.

At the beginning of the capacitation, most of the decapacitation factors are removed (50–75% of the spermadhesins AQN-1, AQN-2, and AQN-3 and 90% of the spermadhesin AWN) [50]. The SpM is destabilized and the cholesterol becomes more accessible to the lipid-binding components of the sow's reproductive tract [42] or to the fatty acid-free bovine serum albumin (FAF-BSA) in the in vitro capacitation systems [13, 33].

In the oviduct sperm suffer a hypoosmotic shock and are exposed to high concentrations of bicarbonate and calcium [70, 71]. This osmotic change produces sperm swelling, and RVD is activated, with the subsequent exit of $[K^+]i$ and $[Cl^-]i$ and activation of the Cl^-/HCO_3^- exchanger, involved in both RVD [46, 53] and sperm capacitation [72, 73]. The output of $[K^+]i$ and $[Cl^-]i$ in RVD, generates water loss and cell volume reduction [29, 53]. These changes in cell volume have been evidenced in *in vitro* capacitation [74].

The activation of Cl^-/HCO_3^- exchanger during the RVD allows the entry of HCO_3^- into the spermatozoa [4, 75] that activates the AC and increases the levels of cAMP [60, 70, 76]. The HCO_3^- in consortium with the FAF-BSA effect the efflux of cholesterol from the SpM, the FAF-BSA functioning as an external acceptor of this lipid [75, 77]. Much of this cholesterol presents a lateral translocation from the equatorial region to the apical region of the sperm head by the activation of the cAMP-dependent PKA [19, 75]. Activation of PKA produces a partial scrambling of PS and PE toward the outer leaflet of the lipid bilayer, in the apical region of sperm head [75]. Parallel, there is a lateral translocation of seminolipids from the apical region to the equatorial region of the sperm head [35, 36]. High levels of cAMP activate the premature opening of Cl^- and K^+ channels, with reduction of $[Cl^-]i$ [58]. This event leads to the entrance of Cl^- to the cell and increase of the CV by the entrance of water [58]. The output of [K+]i and the increase of $[Cl^-]i$ produce a hyperpolarization of the membrane causing the opening of voltage-dependent Ca^{2+} channels [69]. Both $Cl^-$and HCO_3^- were determinants for sperm capacitation, and reduction of Cl^- concentration even in the presence of HCO_3^- suspended the capacitation process [73]. The exchanger channel Cl^-/HCO_3^- works in association with the cystic fibrosis transmembrane conductance regulator (CFTR), which recycles Cl^- to allow the entry of HCO_3^- [73], and the inhibition of CFTR blocks completely the membrane hyperpolarization during sperm capacitation [74].

The entrance of Ca^{2+} activates the tyrosine phosphorylation of proteins [70] necessary for motility hyperactivation and lipid rafts aggregation in the apical region of sperm head, in the presence of FAF-BSA, and HCO_3^- is also activated [37, 75]. Aggregation of lipid rafts is determinant for the binding to the ZP and the acrosomal reaction [13, 40]. These function as molecular signaling platforms where different proteins such as fertilin beta, sp32 precursor, spermadhesin AQN-3, preproacrosin, caveolin-1, and flotillin-1 are involved in the binding to the ZP [37].

4. Cryoinjury in boar SpM

The SpM is the main cellular structure where cryopreservation causes damage; therefore, this must have a special focus [13]. Just at the beginning and during the freezing protocol, the sperm are exposed to mechanical forces such as centrifugation and dilution that favor the depletion of decapacitated factors [78] and the formation of reactive oxygen species (ROS) [79]. The detachment of decapacitated factors destabilizes the membrane and increases the sensitivity to cholesterol efflux [13, 42], and ROS formation produces lipid peroxidation of the highly sensitive membrane by the high proportion of PUFAs and DNA fragmentation [80]. In this respect, there is evidence of cholesterol and PUFA exit from the membrane during freezing, causing loss of membrane integrity and greater peroxidation [10, 16, 17]. During the freezing, it has been observed that protamine 1 and histone 1 suffer determinant structural changes [24].

The cold induces changes in the lipids and proteins of the membrane that determine its functioning [23]. As the temperature goes from 30 to 5°C [23], restriction of the lateral movement of the phospholipids increases, and the membrane passes from a liquid crystalline phase to a gel phase [15, 24]. Because the boar SpM contains a high variety of lipids (PUFAs, saturated fatty acids, cholesterol, and others) with different Tm [9, 16], some lipids tend to jellified earlier than others

[23, 24]. Lipids that are jellified earlier (usually saturated fatty acids) exclude lipids that still maintain a liquid phase [23]. The different groups of fatty acids (saturated and unsaturated), in their different phases (liquid and gel), form order-disorder transitions phases, and the packaging of phospholipids especially in the edges between liquid phase and gel phase is disturbed, forming lipid phase separations [18]. It is worth noting that in human sperm cells that have high amount of cholesterol in the membrane, there is a minimum of lipid phase separation, while these separations occur in an exaggerated manner in the boar SpM that has low amount of cholesterol [23].

The formation of order-disorder transition phases excludes membrane proteins from the phospholipid groups in gel phase toward phospholipid groups that maintain in the liquid phase [18], resulting in loss of membrane-selective permeability by irreversible proteins clustering, disruption of lipid-protein interactions, and translocation or loss of function of ion channels [18, 24, 81]. As a consequence of loss of selective permeability occur: (i) exit of enzymes and cations such as K^+ [15, 18]; (ii) alteration of the water transport and the entry of cryoprotectants such as glycerol [13]; and (iii) influx of Ca^{2+} and HCO_3^- from the extracellular environment [15, 82].

This series of events similar to physiological capacitation, commonly called cryocapacitation, triggering biochemical pathways that result in protein phosphorylation and hypermotility [82, 83]. The main differences between capacitation and cryocapacitation are: (i) cholesterol efflux and the reorganization of membrane lipids during freezing lead to irreversible protein aggregation with loss of function [18]; (ii) the loss of selective permeability of the membrane generates the entry and exit of ions in an uncontrolled manner with differences in the concentration of determining ions [23]; (iii) there are differences in the patterns of phosphorylated proteins [83]; (iv) as the separation of lipid phase is not a reversible process, this possibly affects the aggregation of lipid rafts in the apical region of the spermatic head and therefore the binding to the ZP and the acrosome reaction [13, 18].

5. Biological signals of SpM resistance to cryoinjury in boars

All the molecules reported as freezability marker and those considered potential markers are directly or indirectly related to the physiology of the SpM. For this reason, the following synthesis is plotted according to the location of the molecule in both the extracellular (SP) and intracellular medium (spermatozoa).

5.1. Boar freezability markers

5.1.1. Freezability markers in seminal plasma

Fibronectin 1 is one of the most abundant proteins in the boar SP [52]. This protein possibly interacts with integrins, CD44, and albumin, which suggests its binding to the sperm and a protective action by reducing the effects of oxidative stress [85]. Integrins are proteins expressed in the membrane that connect the extracellular matrix with the interior of the cell and fibronectins, among other proteins, are their ligands [85]. Currently, fibronectin 1 is the only protein recognized as freezability marker [84, 85].

5.1.2. Freezability markers in spermatozoa

Heat shock protein 90 alpha A1 (*HSP90AA1*) has been identified in the spermatozoa flagellum, where it activates the phosphorylation of flagellar proteins in tyrosine residues [86, 87]. In addition, this protein is associated with thermal stress protection [86] and sperm capacitation [88] and is considered a freezability marker in boar [89].

Both *acrosin binding protein* (*ACRBP*) *and acrosin* have been proven as predictors of freezability in boar semen [90, 91]. Acrosin is a proteinase present in the acrosomal domain of sperm, related with the binding and proteolysis of ZP [92, 93]. This enzyme is stored in the acrosome of epididymal and ejaculated spermatozoa in its inactive zymogen or proacrosin [92]. Proacrosin is converted into its mature form, the acrosin [93], during the capacitation, and there is an increase in acrosin activity [94]. In this respect, ACRBP is tyrosine phosphorylated during boar sperm capacitation [95] and intervenes in the conversion of proacrosin [96].

In the capacitation there is a lateral translocation of proacrosin and acrosin, involved in the penetration of the ZP, toward the apical region of the sperm head [94]. This event may coincide with the redistribution of the proteins flotillin-1 and caveolin-1 and the aggregation of lipid rafts in apical region necessary for recognition with ZP and the subsequent acrosomal reaction [37], a phenomenon that may not occur due to alterations in the distribution of lipids in the freezing [13].

High levels of *triosephosphate isomerase* (*TPI*) in refrigerated boar semen correspond with poor freezability [90]. In human semen higher amount of this enzyme in asthenozoospermic samples has been found than in cases of normospermia [97]. In the case of frozen-thawed boar semen, TPI promotes premature capacitation of sperm reducing the freezability [90].

Recently, a relationship of *VDAC2* protein with capacitation in boar spermatozoa has been tested [98]. This association seems to be due to its role in the transport of Ca^{2+} to the mitochondria, which is a determining factor in the capacitation process [99]. In addition, this protein mediates the transport of ions as Ca^{2+}, HCO_3^-, Cl^-, and Na^+ [100] determinants in the processes of capacitation [70, 75] and cell volume regulation in the face of osmotic stress [101]. The condition of freezability marker of this protein [26] can be explained by the occurrence of phenomena similar to capacitation and osmotic stress during freezing [87].

AQP3 and *AQP7* belong to one family of hydrophobic integral proteins of the cell membrane that participate in the transport of water and glycerol [102], essential in the cryopreservation of cells [24]. These proteins are associated with cell volume regulation [45], which is a fundamental process to counteract the osmotic stress caused by freezing [101]. Indeed, AQP 3 and AQP 7 have been previously validated as freezability markers [30].

5.2. Potential boar freezability markers

There is abundant evidence on the leading role of a large group of proteins located both in SP (spermadhesins, Niemann-pick disease type C2 protein (NPC2), lipocalin-type prostaglandin D synthase (L-PGDS); heat shock protein 90 alpha A1 (HSP90AA1); paraoxonase type 1(PON-1), extracellular superoxide dismutase (EC-SOD); and spermatozoon (Cl^- /HCO_3^- exchanger,

Cl^- channel, K^+ channel, AQP, Ca^{2+}-ATPase, ATP-binding cassette transporters, and scavenger receptors), in crucial events of the sperm physiology. This gives rise to intensify studies tending to assess their importance as freezability markers.

5.2.1. Potential boar freezability markers in seminal plasma

Due to the multifunctionality of the *spermadhesins* conferred by their capacity to unite different ligands (heparin, phospholipids, cholesterol, protease inhibitors, and carbohydrates), they participate in several physiological events associated with premature capacitation [51, 103, 104] and with reduction of concentrations of intracellular calcium, sperm survival, motility, and integrity of the mitochondrial membrane [103, 105]. For all the above, it is feasible to think about the possibility of incorporating in the future some of these proteins to the group of freezability markers located in the SP.

The importance of the *NPC2* protein lies in the great affinity of its isoform 19 kDa for the SpM cholesterol [106]. NPC2 is very important in capacitation because it maintains the proportion of cholesterol in the SpM [107] and because it has heparin binding capacity [108]. It is known that during freezing, cholesterol efflux from the membrane leads to cryocapacitation or premature capacitation [10, 16, 17, 109] and that the concentration of 19 kDa protein is higher in semen of high freezability boars and it reduces in 3 h after the ejaculation [110]. These properties and findings suggest a better preventative mechanism against capacitation and serve as a basis to evaluate this protein as a new marker of boar freezability [110].

L-PGDS has high affinity with retinoic acid and retinol [111], two molecules that affect plasma membrane permeability by interacting with phospholipids [112]. Then, L-PGDS could be related to capacitation (acrosome reaction and hypermotility) [113]. L-PGDS is present in the acrosomal membrane in ejaculated spermatozoa and disappears with acrosome reaction [114], and it increases the union in vitro of spermatozoa with the ZP [115], after capacitation [4]. For its affinity for DHA [116] can play an important role in membrane structure and function [117]. This protein is a potential marker of boar freezability because its concentration varies in the seminal plasma of semen with both high and low freezability [110].

HSP90AA1 is a protein considered an intracellular molecular marker for boar semen freezability; it is found in lower quantities in low freezability than in higher freezability spermatozoa [89]. The concentration of this protein increased in seminal plasma of low freezability boars up to 3 h after ejaculation [110], possibly, because of the alteration the plasma membrane integrity during the cooling [118].

The presence in boar semen of the antioxidant enzyme *PON-1* has been reported [119]. The PON-1 influences motility and the SpM integrity because it binds to membrane cholesterol and prevents its oxidation [80]. It is possible that high PON-1 concentration found in the SP of the sperm-peak portion is related with better antioxidant capacities, greater cryotolerance, and lower ROS generation than the post sperm-rich fraction [80]. The above added to the differences detected among boars [120], possibly of genetic origin [121], allows to assume that PON-1 has a potential value as a molecular marker of boar semen freezability. For the case of the *EC-SOD*, it is known that it is in the boar seminal plasma playing an important role as

an antioxidant enzyme in spermatozoa [122]; however, there is a lack of more determinant studies on this protein, which allows establishing its value as a freezability marker.

5.2.2. Potential freezability markers in spermatozoa

The *Cl^- /HCO_3^- exchanger* is part of the solute carrier family 26, number 3 (SLC26A39) [73]. This exchanger has been related to the regulation of CV [46, 53] and has been postulated as one of the possible mediators of the entry of HCO_3^- into spermatozoa and intracellular alkalization during sperm capacitation [37, 75]. The role for HCO_3^- in cholesterol efflux, in the scrambling of phospholipids [75] and the aggregation of lipid rafts during capacitation is clear [37, 75]. Taking into account that in the cryocapacitation membrane reorganization occurs [24, 81], and the need for an adequate regulation of the sperm volume is to counteract the osmotic stress [101], the Cl^- /HCO_3^- exchanger is a molecule that can be tested as a freezability marker.

Individual Cl^- and K^+ channels are the main regulators of the volume of the sperm cell under hypoosmotic conditions [29]. In view of the fact that freezing affects the functionality of the channels by aggregation or translocation and that the spermatozoon suffers a hypoosmotic shock during thawing with consequences on seminal quality [18, 24, 81], the Cl^- channel and the K^+ channel have great importance as possible markers of freezability.

Considering that AQP 3 and AQP 7 have already been tested as freezability markers [98] and that there is an extensive family of *AQP* involved in the transport of water and glycerol [123], aquaporins continue to have great potential as predictors of freezability in boars. This can be supported, also, in the results obtained in bull sperm where differences among individuals have been found on the basis of volume regulation and glycerol permeability [123].

Ca^{2+}-ATPase is an intracellular Ca^{2+} extractor protein located in the head of the sperm that helps regulate the concentrations of this ion [28]. In the boar sperm, when inhibiting this protein, there is reduction of head-to-head agglutination, capacitation characteristic [124]. In knowledge of the entry of Ca^{2+} into the spermatic cell due to loss of selective permeability and membrane lipid phase separations [18, 24, 81], to test whether Ca^{2+}-ATPase levels allow to reduce the cryocapacitation and the differences between individuals in freezing is of relevant importance.

There is little information about cholesterol transporters such as *ATP-binding cassette transporters* and *scavenger receptors*, as well as about the dynamics of reverse cholesterol transport in boar sperm [19]. The low amount of cholesterol in the boar SpM [14, 15] and the efflux of this molecule in the freezing process [16, 17], with the consequences of lipid phase separation in the membrane of this specie [23], can be key events in the study of freezability.

Author details

Julian Valencia and Francisco Javier Henao*

*Address all correspondence to: fhenao@ucaldas.edu.co

Doctoral Program in Agrarian Sciences, Faculty of Agricultural Sciences, University of Caldas, Manizales, Caldas, Colombia

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Section 4

Application of Cryopreservation in Agricultural Researches

Chapter 8

Cryopreservation Protocols for Grapevine Shoot Tips

Zvjezdana Marković, Darko Preiner,
Domagoj Stupić, Željko Andabaka, Iva Šikuten,
Jasminka Karoglan Kontić, Edi Maletić and
Petra Štambuk

Additional information is available at the end of the chapter

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Abstract

Grapevine is an important plant species known worldwide, counting more than 10,000 cultivars of *Vitis vinifera* spread all over the world. There is a strong need for long-term conservation of grapevine genetic resources. With so polymorphic species, it is highly difficult to obtain relevant results considering that cryopreservation protocols were established testing only few very often specific cultivars. Regarding cryopreservation protocols, many factors are influencing the final result. Research articles processing cryopreservation protocols of grapevine are reporting a percentage of recovery, but a broader application of some protocol on a large range of ***Vitis vinifera*** cultivars is limited. How to design an efficient cryopreservation protocol, starting from plant material to the appropriate observation of recovery, will be discussed in this chapter.

Keywords: grapevine shoot tips, cryopreservation protocol, testing of protocol, observation of recovery

1. Introduction

Grapevine is an important plant species known worldwide. Grape is used to produce wines and it is its primary importance. There are many *Vitis vinifera* cultivars; recent research confirmed more than 10,000, but the global market for wine production is dominated by only a few cultivars [1]. Grapevine cultivars do not have equal importance regarding its quality nor potential for wine production. The cultivars are generally classified according to their final production: wine grapes, table grapes, and raisins [1]. Regarding wine cultivars territorially spread within one country, some of them are economically important, whereas some of them have just cultural importance presenting a national viticultural treasure aiming to preserve

biodiversity of the viticultural country. Genetic structure in cultivated grapevines has been shaped mostly by human uses, in combination with a geographical effect [2].

Wine countries are giving a great importance on preservation of grapevine collections and into the development of efficient protocols for long-term maintenance of grapevine biodiversity in vitro, as a safer and more cost-efficient alternative to field collections [3]. Recently in Croatia revitalization of some threatened cultivars is in progress [4, 5]. Mentioned activities are tough and demanding very often with high maintenance costs and the need for additional workers. Thus, a risk of natural hazards to which these collections are exposed is still present. Especially, important long-term storage of plant material can be for small wine countries, like Croatia, because the majority of autochthonous cultivars are economically important, but there are local varieties that present local heritage for some regions or subregions [6]. Particular variety can be highly important for some remote parts within already isolated island (e.g., cultivar Grk at Lumbarda on an island Korčula). One specific example of one cultivar is "Zlatarica from Blato," whereas amphelographic and economic characteristics observed a middle-quality grape variety, but its importance is in conservation of biodiversity of grapevine resources on island Korčula [7]. Also, the big problem can be a virus infection of some variety that has a small population because of the loss of intravarietal variability [8]. Varieties that are intended for wine production need to be conserved in grapevine collections, and there should be a means for its constant evaluation and maintenance regarding their qualitative potential [6]. Wine country like Croatia has many important native grapevine cultivars (Babić, Debit, Grk, Kraljevina, Plavac mali, Plavina, Pošip) with significant wine production. These cultivars are also scientifically explored [9–11]. So, there is a general need in Croatia to make a selection of cultivars that will be maintained in grapevine collections all over Croatia and the rest of the majority of them to be cryopreserved and stored in liquid nitrogen.

The alarming loss of plant biodiversity both in nature and within agricultural systems, for grapevine resources as a polymorphic species, has led us to look for alternatives to in situ conservation. Cryopreservation as a method for long-term conservation exists more than 60 years, and there are experimental protocols already used with a range of crops and geno types, but a broader application of this method has not been achieved in many plant species [12]. Therefore, maybe cryopreservation as a method should be revealed as a useful tool for long-term maintenance of select germplasm [13]. There are examples of gene banks with more or about 100 accessions for potato, cassava, and pear, over the world in different research laboratories [14], but researchers for many plant crops are facing this method, as a means for long-term conservation, as a hardly applicable technique. Often, the initial steps to define protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection are beyond the scope of many plant laboratories [15]. A starting point that is also critical is choice of initial plant material for experiments. In the case of grapevine, this factor was decisive in many cryopreservation experiments [16]. Water status and cryoprotection are the most influential determinants of survival in combination with physiological factors, and that is why crop expert should choose plant tissue that they assume it should survive [13]. The published research mostly relates to *Vitis vinifera*, and the few studies applied to other species show that the protocols need to be improved [17].

In designing cryopreservation experiments, researchers often overlook one major area like culture conditions. In clonally propagated plants, the condition of the plant or the culture can greatly affect the success of a cryopreservation protocol [18].

Research articles dedicated to cryopreservation of grapevine revealed variations in response according to the genotype [19]. The main objective of those articles is to present a cryopreservation protocol with exact result of recovery of some particular genotype of interest for some viticultural area or viticultural country. In so polymorphic crop, as grapevine, it is almost impossible to conclude this method as a technique working or not and whether it is just a matter of genotype.

Some variations in response can be due to the different research laboratories and conditions within. Still in a case of one laboratory that is dedicated to cryopreservation protocols, variations in results with same genotype were observed between years, researchers, and slightly modified culture conditions (data not shown). How to overcome these problems and set up an efficient cryopreservation protocol for the genotypes of interest will be processed in this research note article.

1.1. Establishment of an efficient cryopreservation protocol

Cryopreservation of grapevine first started in 1989 by Ezawa [20], but more detailed cryopreservation protocol for grapevine was reported by Plessis in 1991 [21]. The authors reported survival >72%, but the recovery was not evaluated in the mentioned research [21]. Since then, many techniques of cryopreservation were used by different researchers. The preculture with sucrose was noted as deciding factor for recovery in many of them [21–27]. However, no article revealed how exactly recovery was achieved, and only one research article observed the physiological state of plant material [16]. It is known that the age of mother plants, the size of explants, and some other culture conditions are of paramount importance for in vitro growth of grapevine plants. Also, already in in vitro conditions, great variations between cultivars are reported [28]. Therefore, it is hard to conclude what exactly gave so various results between cultivars. In regeneration in vitro for some cultivars, high genotypic influence is revealed [29–31].

These variations in response according to the genotype are reported in recent review articles of cryopreservation of grapevine [19, 32]. Even if it is noted that any vegetatively propagated species should be amenable to cryopreservation [33], in the case of grapevine, there are examples where explants remain green after rewarming, but do not develop further [24]. The same situation has been revealed in our laboratory, whereas some important native cultivars survived cryopreservation protocol, and they remain green during the following 4 weeks, but unfortunately recovery was not achieved (data not shown).

The most suitable explant for cryopreservation is shoot tip of grapevine, and it is the most used explant in testing of recent cryopreservation protocols for grapevine [16, 17, 34–37]. However, the right time and size of suitable shoot tip are also of a great importance for cryopreservation success as a condition that is adapted to each individual laboratory. Considering that shoot tips of grapevine have the lowest possibility of somaclonal variations, it can be a good choice in testing of protocols. Regarding the cryopreservation protocols recently

established for grapevine, shoot tips were chosen as the most appropriate material for experiments. Experiments of cryopreservation protocols with shoot tips taken from field-grown plants are in a progress in our laboratory (data not shown). This prestep can avoid in vitro culture and simplify the cryopreservation protocol. Furthermore, some of the genotypes are very sensitive to culture conditions and often is difficult to resolve which factors are affecting a weak growth of in vitro plants or inability to regenerate. Unfortunately, to apply cryopreservation protocol, it is necessary to have numerous populations of in vitro material. The situation is more complicated if the success of a particular method requires some preliminary results, where a large amount of already rare plant material will be spent. This is the case of some cultivars from the isolated areas with a small amount of material. Four cultivars from place Kaštela near Split were introduced in tissue culture conditions (2011). Initial growth of plant material was satisfying, but in the period when we should already have adult plants for experiments, the plants start to yellow and browning and gradually decay. Urgent attempts of intervention with various media with higher concentration of growth regulators have been applied. More or less the same situation with same cultivars was repeated following 4 consecutive years with fewer deviations. The material was taken from a virus-infected plant. Also, international cultivars (Cabernet Franc, Cabernet Sauvignon, Grenache, Merlot) were taken from infected vines and introduced in tissue culture conditions in order to test the method of cryotherapy. Cultivars Cabernet Sauvignon and Grenache were very sensitive to culture conditions where initial culture already failed. Cabernet Franc and Merlot grew well, but regular multiplication was not successful enough to give the requested amount of plants to carry out the experiments [38]. This problem has been investigated on a cell level with "Gala" apple cryopreserved shoot tips [39]. They found cell membrane damage and alternation in mitochondria which caused a slower shoot proliferation in cryopreserved shoot tips. Even if our study does not include cryopreservation, this can be due to the virus infection that causes a weak growth in tissue culture conditions of infected cultivars tested. How to obey this problem for the cultivars of interest will be investigated in the following experiments in our laboratory.

Choosing the right method for testing a cryopreservation protocol is necessary to observe laboratory capacities and, in general, to define the final aim of the procedure. The current situation revealed a lot of tested protocols, significantly different, resulting without the broader application of this technique for grapevine. How and when will this important method in viticulture come to life depends on how it is presented in scientific and expert area. Testing of the cryopreservation protocol is a too demanding procedure if we want to only reach some good percentage of survival or to get fast results. The final aim should observe the cultivar of interest in a given condition for long-term conservation, and only then this type of experiments is reasonable. In the case of just testing, the protocol with representative data is an example of this method made on irrelevant cultivars or rootstocks that have reached high percentages of regeneration. This is a method of scientific approach that should not be performed with the aim of not giving the wrong picture of the method in general. Of course, some non-important cultivars and rootstocks should be tested because of their national importance and the need for long-term preservation that has been already mentioned above. From all experiments made in one laboratory, "model cultivar" should arise from all thoroughly tested steps from

choosing the appropriate material for experiment, defining a duration of exposure to cryoprotectant solutions to the already selected most appropriate medium for regeneration. Once the protocol is set up for cryopreservation as the "standard" one, then it could be concluded how many laboratories and which one advanced in that area with cryopreservation protocols. That assume that all culture condition requirements are suitably adjusted undoubtedly not affecting the procedure of cryopreservation protocol. When exact protocol is adopted, it can be tested in several different laboratories on the same cultivar with a comparison of obtained results.

Knowledge of growth, development, and regeneration of grapevine in tissue culture conditions is a crucial factor in achieving success. From the beginning of tissue culture on grapevine, there are many protocols in use, but there is only limited use in viticulture.

It was reported that in vitro establishment was achieved with nodal segments taken outdoor from just mature canes of 2-month old [40]. Alizadeh [40] also reported that micropropagation will inevitably be necessary for mass propagation in different horticultural crops. Chee and Pool [41] first designed a large scale of micropropagation protocol where in 8 weeks one person can deliver 2000 vines of 6 cm high and with six to nine nodes. This presents a grown plant of grapevine in vitro. This definition should be a copy in defining recovery after cryopreservation of grapevine in vitro plants. The most similar results were obtained in our in vitro laboratory, when achieving in vitro grown plants, if the protocol of micropropagation is working, is needed at least 2 to 3 months. To record the recovery or regeneration after cryopreservation, grown plants should be achieved; otherwise, it is not clear enough does regeneration has been achieved. Considering that laboratories are describing recovery differently, any other regeneration of grapevine in vitro plants can be questionable.

1.2. Preculture of explants

In the case of cryopreservation protocols for grapevine, preculture means exposing explants, which are intended for cryopreservation protocol to solutions with sucrose or some other additives. The accumulation of endogenous cryoprotectants such as sugar and sugar alcohol may increase the stability of membranes under conditions of severe dehydration [13]. It is noted that the most frequent cryopreservation protocols applied on grapevine include preculture, no matter which method has been applied [19]. The main aim is to induce tolerance of in vitro stock cultures and explants to dehydration and subsequent freezing in LN.

Protocols initially developed for grapevine, including the method of encapsulation-dehydration, allude the treatment of explants with increasing the sucrose contents in a medium [21, 34, 36, 42]. However, in order to increase the level of regeneration, researchers explore different types of preculture. In recently developed protocols with the method of droplet vitrification [16], preculture was a basal solid medium with 0.1 M sucrose concentration. In his first improvement of droplet vitrification method, Pathirana et al. [17] increase the level of regeneration with addition of 0.1 mM of salicylic acid in pretreatment. More detailed research revealed that preculture medium can be composed with more components: 0.3 M sucrose, salicylic acid, glutathione and ascorbic acid [43].

In our laboratory, research made on the influence of preculture on survival after cryopreservation revealed that explants should be cultivated in a form of microcuttings on the medium with addition of growth regulators [36]. Although the procedure of preparation of microcuttings is homogenizing the material where all axillary buds take over the role of shoot tip, addition of benzyladenine (BAP) in a medium can improve regeneration [16] after cryopreservation that was reported. At any rate for grapevine, explants themselves in a size of 1 mm, the greatest size for survival, can not survive an extremely stressful process of freezing, without the previous preparation.

In our case, a preculture with 0.1 M sucrose in a medium for 24 hours, just before procedure of cryopreservation [36], resulted as the greatest for grapevine shoot tip survival after cryopreservation and was applied in the following research [16, 37]. The importance of preculture arises from the fact that direct immersion in liquid nitrogen of one *Vitis vinifera* cultivar resulted without survival after cryopreservation [44] even if the report is quite old.

Some presteps or addition of some steps in the standard protocol of cryopreservation has been tested. Improvement of plant regeneration in cryopreserved kiwi fruits was revealed [45]. Cold acclimation of donor plantlets at 4°C for 2 weeks followed by sucrose preculture of shoot tips and supplementation of ascorbic acid (0.4 mM) in all media throughout the procedure registered 40% regeneration after cryopreservation. Cold-acclimated plantlets and ascorbic acid pretreated shoot tips exhibited severe plasmolysis and some disruption of membrane and vacuoles. This study revealed that only those cells that have been dehydrated and plasmolyzed can withstand cryopreservation without vitrification [45]. This type of experiment design is highly needed in cryopreservation of grapevine.

1.3. Role of genotypes

In a recent review article about cryopreservation of grapevine, it was noted that this method is highly genotype sensitive. Even the previously thoroughly tested protocols applied on different grapevine cultivars revealed significantly various results [25], they observed regrowth rates from 30% in Teleki 5BB (*V. berlandieri* × *V. riparia*) to 86.7% in Merlot (*V. vinifera*). Whenever more than one genotype tested in the success of cryopreservation protocol on grapevine shoot tips, a strong genotype effect was reported [17, 23, 37, 46, 47]. In this overview it was noted that significantly more *Vitis vinifera* cultivars were tested in comparison with rootstocks and hybrids. Considering the general need for conservation of grapevine genetic resources [19, 32], *Vitis vinifera* cultivars are rather a better choice. However, regarding the main aim of the method, each research laboratory is trying to find solution for the cultivars of interest where consequently so many cultivars were tested through this method.

Cryopreservation of grapevine shoot tips has been tested with different methods of cryopreservation. Firstly, Ezawa et al. [20] reported two-step cooling with three *V. labrusca* genotypes reaching 96.7% of survival, respectively. Esensee and Stushnoff [44] tested one *Vitis vinifera* cultivar without any survival by direct immersion in liquid nitrogen, as reported earlier. Neither two-step cooling nor direct immersion in liquid nitrogen was tested with grapevine shoot tips afterward. Combination of encapsulation-dehydration and two-step

cooling was made on nine *Vitis vinifera* cultivars [22, 46, 47], and even in different research laboratories, there was a quite stable rate of recovery reaching around 30%. With a method of encapsulation-dehydration, six *Vitis vinifera* cultivars were tested reaching 37–63% of recovery [23, 34, 36, 42, 48]. Encapsulation-vitrification as a method was tested with only one hybrid (*V. berlandieri* x *V. riparia*) with no specified number of survival [49]. The most used method for grapevine shoot tips was vitrification and more recently droplet vitrification. Regarding vitrification various results were obtained with different types of genotypes reaching from 30 to 86.7% of recovery [25, 27, 34, 48, 50–52]. Even if the method of droplet vitrification was designed to achieve some improvements, extremely low rate of 6% of recovery was reported [17], and high percentage (76%) of recovery was noted [53]. From the mentioned results, it is difficult to conclude which cultivar or genotype is adaptable to cryopreservation protocol in order to test the protocol. Results clearly revealed that there is still no "standard protocol" for grapevine shoot tips effective in each research laboratory. Thus, a major problem arises from the fact that different cultivars are responding differently, and in many cases to achieve high percentage of recovery, a cultivar of interest should be previously tested. From our point of view, there should be one cultivar responding very well in tissue culture conditions that should be tested in each cryopreservation laboratory. The proposed solution is the only scientific way we could observe efficiency and repeatability of cryopreservation laboratories.

2. Conclusion

A general perspective for cryopreservation of grapevine is questionable considering the long period needed for adjustment of the protocol. Also, in adjustment of the protocol to the one cultivar, we are in a large risk to waste the time and money, if tested protocol does not work on the range of cultivars. In the review article [19], it was observed that more than 25 reports were made on grapevine shoot tips; however, no wide range of application was made, even needed for grapevine genetic resources. With here listed and suggested guidelines, we could surely take the opportunity to make a common research and with joint forces come to the solution.

There is no existing a grapevine gene bank fo far, so we assume that all grapevine cryopreservation specialists have the same interest and that is the application of tested protocols to a greater number of cultivars. Consequently, some type of association testing grapevine cryopreservation protocols, at least with regard to material, variety, and tested protocols, should be united. The highly demanding protocol for grapevine that will give more or less the same results on one grapevine cultivar, chosen as a "model cultivar," through cryopreservation research laboratories around the world is surely the beginning that all trustworthy laboratories should aspire.

Hopefully, that part of our proposals will start to be implemented in the near future, and we will continue to work intensively on developing a single variety protocol and further testing of cultivars of interests that need long-term preservation.

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Author details

Zvjezdana Marković*, Darko Preiner, Domagoj Stupić, Željko Andabaka, Iva Šikuten, Jasminka Karoglan Kontić, Edi Maletić and Petra Štambuk

*Address all correspondence to: zmarkovic@agr.hr

Department of Viticulture and Enology, University of Zagreb, Zagreb, Croatia

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Cryopreservation has many biotechnological applications in different fields. This has led to an increase in importance of cryobiology as a science that examines the effect of ultra-low temperatures on cells, tissues, organs and organisms and also the freezability of these structures, while maintaining their viability. Nowadays it is well known that this form of biotechnology can be used to solve a lot of problems such as human infertility, life threatening diseases, preservation of gametes and DNA and also biodiversity conservation.

Cryopreservation Biotechnology in Biomedical and Biological Sciences describes principles and application of cryopreservation biotechnology in different research areas and includes seven chapters that have been written by experts in their research fields. The chapters included in this book are thought to improve the current understanding of the different areas of using cryopreservation biotechnology.

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