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Goral Gandhi
Editors

Vitrification in Assisted Reproduction

A User's Manual

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Foreword

A flurry of outstanding inventions has brought Assisted Reproductive Technology (ART) to the remarkable place where it stands now. Over five million healthy babies were born thanks to these highly imaginative and sophisticated methods. Yet, several major leaps have characterized the gradual development of ART over the last three decades. For instance, the introduction of techniques such as transvaginal oocyte retrieval and intracytoplasmic sperm injection (ICSI) totally revolutionized the capability of ART methods to offer highly effective solutions to infertility problems. *Vitrification* undoubtedly presents the latest giant leap in ART, totally revolutionizing the field of oocyte and embryo cryopreservation.

The mystical poet and philosopher *Khalil Gibran* is quoted as saying "*Progress lies not in enhancing what is, but in advancing toward what will be*." It seems that the new vitrification solutions, so eloquently detailed in Dr. Gautam Allahbadia and Dr. Goral Gandhi's new stimulating book, clearly demonstrate how this new field is moving us toward new capabilities and is opening the doors for a better future to our patients, who all strive to gain better access and higher success rates in ART.

This exciting volume presents a real comprehensive "User's Manual" for this contemporary field. Thus, reading this book will not only enable the reader to grasp the physiology and science behind vitrification but will also assist him in rapidly and safely implementing these innovative but crucial lab techniques in his own IVF lab.

The book offers a wonderful overview on vitrification by one of the true pioneers of this filed, Masashige Kuwayama from Japan. This is followed by a vibrant description of the historical development of novel vitrification techniques by another master of this field, Luis Ruvalcaba of Mexico.

Detailed chapters offer many practical "tips and tricks" as well as trouble shooting clues to carefully incorporating the wide array of indispensable methods for vitrifying oocytes, sperm cells, pronuclear embryos, day 2–3 human embryos, blastocysts, and embryonic stem cells.

Other important topics effectively described in this effortlessly read guide include various contemporary methods for vitrification, ovarian tissue vitrification for fertility preservation, nitrogen vapor shipment of vitrified cells, and oocyte banking. These chapters were written by many highly acclaimed experts such as Noriko Kagawa of Japan and Gabriel Dalvit from Argentina.

Vitrification was introduced on a worldwide scale only recently but has now become an essential technique for any successful modern ART Unit. This of course makes reading this brilliantly written book *a true must* for any aspiring ART specialist, physician or embryologist, novice, as well as highly experienced professional.

Tel-Hashomer, Israel Daniel S. Seidman, MD, MMSc

Preface

> I did then what I knew how to do, now that I know better, I do better. Maya Angelou

At leading IVF clinics, the topic of vitrification of ART has always been at the forefront of clinical practice, given the promise of the technique and the potential to influence ART. This topic has seen increasing interest among practitioners and evidenced by the many hands-on workshops and conferences over the past 5 years. This book is the outcome of such sessions and, we hope, will illuminate the journey of professionals keen to understand the principles and techniques of vitrification as relevant to ART.

The first ever workshop held in India by us was attended by participants from around the world including one professional who had traveled all the way from Hawaii. Over the years, we have had professionals from several countries coming over to India to participate in these sessions. Interaction and feedback received from them outlined and defined to a great extent the contents of this book.

It is well known now that vitrification has dramatically improved the outcomes of cryopreservation and in turn the success rate of IVF using frozen embryos and gametes. Vitrification in cryopreservation has found such acceptance from embryologists far and near that it can no longer be considered "emerging," much less questioned. The versatility of vitrification techniques allows for cryopreservation of both embryos and gametes with equal measures of success, thereby changing the way ART has been traditionally practiced. Increasing demands for fertility preservation in women particularly in cases of malignancy and occasionally for social reasons can now be clearly met owing to vitrification. The book covers topics from basic fundamentals of vitrification techniques, vitrification of embryos, blastocysts, gametes, ovarian tissue, and even human embryonic stem cells. The book also discusses topics such as oocyte banking and transportation of vitrified gametes and embryos, which are the demands of a modern-day ART clinic.

Our contributors from across the globe are experts in their own field and have drawn from their own vast experiences to give our readers a panoramic overview of the field and some fascinating details from their practice. Our heartfelt thanks to our contributors Luis Ruvalcaba, Soumya Ramesh, Sakina Kagalwala, Noriko Kagawa, Pankaj Talwar, Gabriel Dalvit, and Pratik Tambe for their efforts and excellent contributions.

In this field, much valuable work has already been published; but present writings are addressed rather to trained practitioners than to beginners. It is our fond dream to present advanced vitrification techniques unencumbered with hard words and difficult principles even if it is not altogether an easy subject. We have endeavored to present in a simple way the terminology and principles that enhance the difficulty which a beginner is likely to experience in grasping and assimilating the facts while doing away with confusing details. The leading object of this book is to be both as scholarly and as practical as possible. We hope that you will enjoy reading the book as much as we have enjoyed writing and compiling it.

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Dr Mrs Asha Singhal was my earliest guide, introducing me to the field of embryology when it was in the nascent stages and largely unknown to many. It was her advice and direction which enabled me to be a participant in this field.

I thank Masashighe Kuvayama for being a wonderful person and an exceptional teacher. My own understanding and practice of vitrification is owed completely to him.

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Mumbai, India — Goral Gandhi

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1 Vitrification: An Overview

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Vitrification • Vitreous state • History • Phase diagram • Cryoprotectant

Introduction

The introduction of newer technologies has led to an increased significance of cryopreservation in assisted reproductive programs. Cryopreservation is routinely carried out using the slow-freezing protocol, which is a well-established procedure. However, there are several disadvantages to the slow-freezing protocols, formation of intracellular ice crystals causing considerable damage to the cells.

The hazards of ice crystal formation are significantly reduced by rapidly cooling the biomaterial to low temperatures at sufficient rates to produce an amorphous solid. This alternative was proposed by Luyet in the 1930s and is called vitrification [1]. Vitrification is the kinetic process by which a liquid solidifies into a glass (derived from "vitri," the Greek word for glass). A glass is a liquid that is too cold or viscous to flow. Vitrification does not have any of the biologically damaging effects associated with freezing because no appreciable degradation occurs over time in a living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biological systems.

History of Vitrification

1930 – Walter Stiles [2]
The death of plants as a result of exposure to temperatures low enough to bring about freezing of the water in the tissues is caused by the formation of ice crystals in the protoplasm, which bring about a disturbance in the relations between the disperse phase and the dispersion medium, which cause aggregation of the disperse phase. This is usually irreversible; on thawing, the original colloidal system of the living protoplasm is not reformed, and the protoplasm in consequence is no longer living.

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He proposed that by very rapid freezing, it is possible to form a finely crystalline or even amorphous mass that might give the original system again on thawing.

1937 – Basile J. Luyet [1]

In his experiment on vitrification of organic colloids and of protoplasm, he found that:

> Ice formation is not compatible with the survival of living system and ought to be avoided during freezing. There are 2 intrinsic factors that control the production of the vitreous state; they are the velocity of crystallization and the size of the zone of crystallization temperatures. A third factor is extrinsic and depends on the method employed- it is the cooling velocity. The essential problem of the vitrification technique consists of obtaining a cooling velocity sufficient to prevent the formation of crystals.

The idea of cryopreserving cells within a volume rendered glassy by rapid cooling is generally credited to Luyet.

1940 – Luyet and Gehenio [3]

> Good vitrification is not injurious, there being no molecular disturbance, while an incomplete vitrification or devitrification and, a fortiori, crystallization, are injurious to the extent that they disrupt the living structure.

1968 – Rapatz and Luyet [4]

They successfully vitrified human red blood cells – they showed that red blood cells could be cooled to liquid nitrogen temperature (−196 °C) without ice formation. Freeze-fracture electron micrographs showed decreasing amounts of ice in samples with increasing concentrations of glycerol until there was no visible ice.

1976 – Tokio Nei [5]

Nei reproduced Rapatz and Luyets results of vitrified red blood cells.

> As a cryotechnique for electron microscopy, the addition of 30 % glycerol and ultrarapid freezing at 10^5 °C/min are minimum requirements for inhibition of ice formation and the prevention of the corresponding artifacts in erythrocytes.

1980 – Bruggeller and Mayer [6]

They published the first reproducible demonstration of the vitrification of pure liquid water and dilute aqueous solutions. Dilute aqueous solutions in contrast to concentrated aqueous solutions behave similar to water and separate during freezing, even with the highest cooling rates available, into pure ice and concentrated solute.

1984 – Boutron and Arnaud [7]

Red blood cells were cooled to −196 °C in buffered solutions containing 10, 15, 20, 30, or 35 % (w/w) 1,2-propanediol or glycerol. For the same concentrations, the minimum occurs at much lower cooling rates with 1,2-propanediol than with glycerol, in agreement with the better glass-forming tendency of 1,2-propanediol solutions. Thus, survival seems to be closely related to the glass-forming tendency at the survival minimum and at higher cooling rates.

1984 – W. F. Rall, D. S. Reid, and C. Polge [8]

The analysis of slow-warming injury of mouse embryos by cryomicroscopical and physiochemical methods showed subsequent rapid cooling and rewarming.

They concluded that slowly frozen embryos survive as a result of intracellular vitrification.

1984 – G. M. Fahy, D. R. MacFarlene, and C. A. Angell [9]

"Vitrification as an approach to cryopreservation."

They described vitrification solution that was both capable of permitting vitrification at 10 °C/min and compatible with a 90 % functional recovery of the renal tissue.

Fahy recognized that aqueous solutions with suitable concentrations of cryoprotectants could be vitrified by supercooling and rewarming at practical rates through the metastable nonequilibrium zone between the ice melting temperature and the glass transition temperature. Interestingly, crossing below melting temperature meant that the required cryoprotectant concentrations for vitrification by supercooling were actually lower than the concentrations seen by cells at the end of freezing. However the concentrations were still higher, and therefore more toxic, than those used at the beginning of freezing methods. Numerous details of cryoprotectant composition and introduction of protocols were refined by Fahy to make it possible for a living tissue to tolerate vitrification under practical conditions.

1985 – W. F. Rall and G. M. Fahy [10]
"Ice-free cryopreservation of mouse embryos at −196 °C by vitrification." They established vitrification as a viable and potentially a general alternative to freezing. Fahy proposed nonequilibrium vitrification as a way to cryopreserve organized tissue, especially organs, without structural damage from freezing.

Vitrification

Vitrification refers to a physical process wherein solidification of a liquid solution occurs to form a glass-like vitreous state not by the ice formation but by extreme elevation in viscosity during the process of cooling. While being cooled to extremely low temperatures, this liquid will meet its glass transition temperature at which the change from the liquid phase to solid phase occurs. When in a glass state, a solution is still technically liquid but is too cool to flow [11]. Indeed, throughout cooling at lower and lower temperatures, the molecules will remain in the same disorganized way as they are when in a liquid state. Nevertheless, the physical properties of the "liquid" are now comparable to those of a solid. Therefore, the molecules are held in the same pattern through the whole process. The "solid liquid" obtained is defined as glass.

Phase Diagram

A phase diagram shows conditions at which thermodynamically distinct phases can occur in equilibrium. Examples of different phases of water that can occur in cryopreservation are liquid, ice, and glass. In a phase diagram, lines of equilibrium, also called phase boundaries, are shown that mark conditions under which multiple phases can exist in equilibrium. Figure 1.1 is a hypothetical phase diagram for the changes occurring during freezing with a cryoprotectant [12].

In this figure, T_t (also called T_m) is the equilibrium freezing temperature of the melting point curve, T_h is the homogeneous nucleation temperature before the solution actually nucleates (i.e., begins to freeze), T_g is the glass transition temperature at which supercooled liquid vitrifies, and T_d is the temperature at which devitrification occurs.

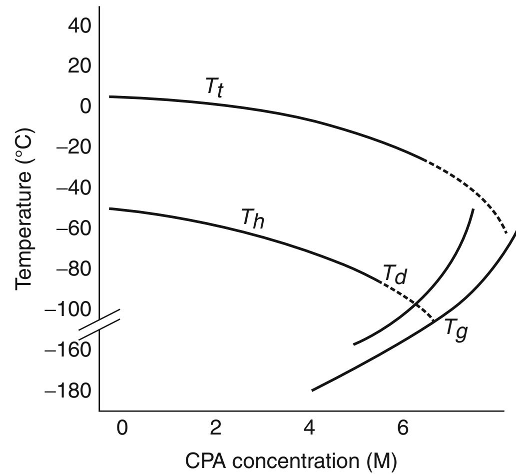

Fig. 1.1 Phase diagram of hypothetical aqueous solution of cryoprotectants (Reprinted from Quinn [12], with permission from Cambridge University Press)

Crystallization of the solvent (e.g., water) is possible in the region between T_t and T_h. The opportunity for vitrification depends on CPA concentration and temperature and lies along T_g, the glass transition curve. To make a glass with a minimum of embedded ice crystals, high concentrations of CPA must be used and must be cooled as quickly as possible to a temperature below T_g. Devitrification (T_d), crystallization in vitrified system, occurs when the glass warms above T_g through the growing of embedded ice crystals. This means that thawing should also be done as quickly as possible. The described elements of the phase diagram are relevant because efficient vitrification involves either a phase transformation or its avoidance.

Vitreous State

At temperature below T_m/T_t (melting temperature), the rate of entropy reduction of a supercooled solution is greater than a corresponding frozen solution. If the trend were to continue to even lower temperatures, the supercooled solution would eventually have less entropy than the corresponding mixture of concentrated solution

and ice. Such a situation, however, would be in violation of the known principles of thermodynamics because the crystal has the lowest entropy possible for a given substance. Kauzmann discussed this paradox and elucidated a rationale for its resolution [13]. With sufficient reduction in temperature, thermal energy becomes insufficient to drive rotational and translational motions. Water molecules become trapped in local energy wells due to the removal of internal energy, and the entropy therefore stabilizes and remains above that of the ice phase. This event is referred to as the glass transition.

The vitreous state is essentially a solidified, amorphous liquid state obtained by specific conditions of cooling and solute concentration that inhibit ice nucleation and growth. At low temperatures, the viscosity of the solution becomes so high that the sample gets immobilized. This sample then takes on the characteristics of a solid.

So, vitrification is the direct conversion of a liquid state into glass, bypassing or rapidly passing through the critical temperature of ice formation (no phase transition).

Thermodynamics of Vitrification

Gibbs free energy is the energy associated with a chemical reaction that can be used to do work. The free energy of a system is the sum of its enthalpy (H) plus the product of the temperature (Kelvin) and the entropy (S) of the system:

$$G = H - TS$$

To determine whether water in aqueous solution is thermodynamically stable in the liquid or solid phase at a specific temperature, the difference in Gibbs free energy (ΔG) between each phase at that temperature can be calculated. As the temperature of an aqueous solution is lowered below its melting temperature (T_m), G ice becomes less than G liquid water, and ice becomes a more stable state. G increases with increase in temperature for water and ice. However, ΔG water increases faster than G ice, and it is the driving force for crystallization. Despite ΔG (G ice – G water) becoming negative as cooling passes below T_m, immediate crystallization rarely occurs. Crystallization is initiated by random aggregations of water molecules to form small volumes of the new phase (ice crystal nuclei). Creation of a stable nucleus needs overcoming an energy barrier associated with the formation of the liquid–crystal interface. Once a stable nucleus is formed, crystallization continues. Crystal growth is a kinetic phenomenon in which molecules diffuse from liquid phase to the interface and rotate to get incorporated in a crystal. Nearby T_m, molecular mobility is higher; hence, crystal growth rate is highest near T_m. At lower temperature, the rate of molecular motion slows down and thereby reduces ice nucleation rate. Therefore cooling very quickly (during warming) minimizes the time available for nucleation and crystal formation. Also, it diminishes the solute concentration necessary to keep it in vitreous state.

Probability of Vitrification

The molecular configuration of the supercooled liquid ($T \geq T_g$) is the same as that of the glass ($T \leq T_g$). Hence rapid cooling is necessary to prevent the supercooled liquid molecules from reorganizing into a regular (e.g., lattice) configuration. Vitrification is a second-order phase transition. Hence, by definition, the specific volumes of both phases (near T_g) are identical, although the thermodynamic property values (i.e., heat capacity, coefficient of thermal expansion) are not [13].

The difficulty in successfully vitrifying a material lies in reaching its glass transition temperature T_g prior to crystal formation. Hence reducing the distance between T_m and T_g by increasing the solute concentration and cooling at ultrarapid rates increases the probability that a given liquid will form a glass.

At high concentrations of CPA, the glass transition temperature (T_g) increases, but devitrification can occur during warming (T_d). It is theoretically possible to cool and produce a stable glass without ice formation, but this requires CPA concentrations in excess of 80 % w/v, which are very toxic to cells.

The challenge with the use of vitrification in human-assisted reproduction is to develop a practical approach that will help in achieving high enough cooling and warming rates along with the lowest level of toxic cryoprotectant combinations that ensures a safe vitrified state under the given cooling and warming parameters. Three key factors influence the probability of successful vitrification: cooling and warming rates, the composition of the CPA solution, and the sample volume [14]. The requirements and relationships for conditions to achieve satisfactory vitrification in the area of mammalian ART are well displayed in the pseudo-equation of Yavin and Arav [14]:

$$\text{Probability of vitrification} = \frac{\text{Cooling and warming rate} \times \text{viscosity}}{\text{Volume}}$$

Increasing the CPA concentration and decreasing sample volume will each increase the probability of vitrification. Volume reduction, along with a subsequent increase in the rate of cooling, also results in the lessening of concentration of the cryoprotectants, which eventually reduces their potential toxicity. All these conditions when looked into carefully cumulatively add up to give excellent survival rates on warming.

Cooling and Warming Rates

Very slow or very high cooling rates can damage cells through osmotic effect and pH changes. Very slow cooling rates tend to encourage extracellular ice formation, while the very high cooling rates tend to promote intracellular ice formation: both could be lethal to cells. With vitrification, however, the cooling and warming rates are sufficiently fast to never allow ice formation either upon cooling or upon warming. Most other physiological changes that occur with slow cooling cryopreservation, such as osmotic effect, solution effects, and dehydration, are also eliminated by vitrification as during the process the entire solution remains unchanged except for the transition of water from a liquid to glassy phase [15].

Once a biomaterial has been vitrified successfully, it must be stored at temperatures below T_G to encourage stability. It has been noted that rapid warming rates are a prerequisite to successful vitrification as it prevents the vitreous water present in cells from crystallization at the time of warming [16]. Devitrification, crystallization is possible since at temperature greater than T_g, the crystalline phase is more stable than the amorphous solid.

The Composition of CPA Solution

The composition of vitrification solution is an important aspect to be considered given the high concentrations of cryoprotectants used. Several different mixtures of cryoprotective agents have been tested as potential vitrification solutions. Different CPAs have different permeability and are also temperature dependent. The toxicity of a CPA is correlated to its concentration, the time of exposure, and the temperature. Using CPAs at room temperature or lower rather than 37 °C may decrease their toxicity. Nevertheless, most of these solutions are toxic to the cells, particularly the low molecular weight agents. The toxicity depends on different parameters, such as the time of exposure, and concentration.

There are now two main categories of cryoprotectants, the penetrating and non-penetrating ones. Penetrating cryoprotectants are used to protect the cells and tissues by replacing some of the water they contain and thus increasing the viscosity in the cells, preventing the formation of ice crystals, and making the vitrification process easier by inducing the transition to the glass state [17]. The non-penetrating protectants prevent water from flowing out of the cells and the occurrence of osmotic damage by making the membrane less permeable [17]. Some natural antifreeze proteins can be used in addition to these cryoprotectants; their role is to inhibit ice nucleation.

Measuring the glass-forming efficacy of the CPAs that compose the vitrification solution has revealed that an excellent vitrification solution can be formed by a combination of a balanced concentration of a strong glass former such as dimethyl sulfoxide and a weak glass former such as ethylene glycol, acetamide, or formamide. This favors cell viability [6].

It can be beneficial to use additive combinations of CPA to maximize survival. For example,

when cryopreserving mammalian preimplantation embryos, a cell-permeating CPA such as DMSO may be used in combination with a sugar such as sucrose [18]. Beyond the simple colligative effect, this sugar may act in several ways. As a non-permeating solute, the sugar will act as an osmotic agent, reducing the water content in the intracellular compartment even before ice formation begins. The sugar being a solute with a high viscosity at low temperatures will increase overall viscosity of the mixture during freezing and facilitate the transition to a glassy state.

Conclusion

Vitrification involves avoiding the formation of ice by using such a high cryoprotectant concentration that the system becomes so viscous during cooling that it forms a glass and does not crystallize as ice. Cellular materials that can be vitrified can escape solution effects, the dangers of intracellular injury, and the other damaging effects of slow cooling. Vitrification has been increasingly used as a method of cryopreservation for reproductive cells and tissues, but many issues still remain to be addressed. The challenges include the development of a less cytotoxic vitrification solution and of a safe vitrification device in order to have vitrification techniques considered as a standard clinical laboratory procedure. The concentration of CPA can be reduced by using very rapid cooling and warming because this inhibits the formation of ice crystals and this approach has been successfully used with small volumes of cell suspension, but in the case of tissues, the rates of change of temperature that are possible are restricted by the physical circumstances – relatively bulky samples.

Advantages of Vitrification

1. Rapid vitrification/warming reduces the time of cryopreservation procedure.
2. Utilizes higher concentration of CPA, which allows shorter exposure time to the CPA.
3. Using small volumes for freezing improves the rate of freezing.
4. Eliminates crystallization.
5. Minimizes osmotic injury.
6. Simple protocol.
7. Eliminates the cost of expensive programmable freezing equipment and their service and maintenance.

Variables Influencing Effective Vitrification

1. Cryoprotectant – type and concentration of cryoprotectant.
2. Temperature – temperature of vitrification solution at exposure.
3. Time – duration of exposure to the final cryoprotectant before plunging into liquid nitrogen.
4. Volume – the smaller the volume of the vitrification solution, in which the cellular material is placed, the faster the cooling and warming rates that can be achieved and the lower the concentrations of CPAs that can be used.
5. Rate of cooling and warming – high rates of cooling and warming avoid ice crystal formation.
6. Carrier – type of carrier device.
7. Experience of the operator.

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2 Vitrification Solutions: Historical Development

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Keywords

Vitrification history • Cryoprotectants • Permeating cryoprotectants • Nonpermeating cryoprotectants • Vitrification techniques

Vitrification, a word originating from the Latin "vitreum" or glass, is a process that produces a solidification of cells similar to glass, which prevents the formation of crystals during cooling [1]. Usually, the transformation is obtained by rapid cooling of a liquid that due to certain chemical reactions causes it to transform into the consistency and characteristics similar to glass.

Over the past decades, more than 800 articles have described cryopreservation of oocytes and embryos by vitrification, the reversible transition of liquid into an amorphous noncrystalline glass. Vitrification presumably requires high concentrations of cryoprotective additives and very high cooling rates to produce the glassy state. Vitrification of reproductive cells and tissues, nowadays, has been achieved by suspending them in solutions containing 15–30 % permeating cryoprotectants plus 18 % saccharide and cooling very small samples at rates of >10,000 °C/min [2].

The methods and protocols for cryopreservation – slow cooling or rapid cooling protocols – both satisfy the fundamental cryobiological principles for reduction of damage by ice crystal formation during cooling and warming. Both methods include six principal steps:

- Initial exposure to the cryoprotectant (intracellular water has been removed by gradual dehydration)
- Cooling (slow/rapid) to subzero temperatures (−196 °C in storage)
- Storage at low temperatures
- Thawing/warming by gradual rehydration
- Dilution and removal of cryoprotectant agents and replacement of the cellular and intracellular fluids at a precise rate
- Recovery and return to a physiological environment [3]

The procedures to achieve vitrification have gone through a historical process of evolution, and the first reports of cryopreservation of tissue cells date to 1766, when Spallanzani published the first cryopreservation of sperm in snow and the capture of mobile spermatozoa.

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Biologists established some early concepts of cryoprotection in the late nineteenth and early twentieth century, by studying the freezing cold hardiness and frost resistance in the environment mainly in plants. For example, Hans Molisch in the 1890s considered freezing in plants by direct microscopy using a cryomicroscopy [4]. Molisch was aware that the composition and concentration of substances in the cytoplasm of plant cells essentially dictate survival or death after freezing. The importance of sugars as cryoprotectants was clearly recognized by Maximov [5] in the 1900s. Similar observations on the biochemistry of resistance to the cold in plants and insects continued in the next 30 years, but it was not until 1938 and 1945 that scientists found that sperm, frozen and stored at a temperature of −160 °C, could survive. Polge performed the first study of freezing semen in the year 1949 using glycerol as a cryoprotectant to prevent damage from the freezing process [6]. This led to the widespread application of cryopreservation in medicine, biotechnology, and vegetable and animal improvement. In the early years, these protective chemicals were called "cryophylactic" or "moderators of solute" [5].

Subsequently, in 1948, Chang et al. [7] published the first cryopreservation techniques and thawing of rabbit embryos, in which a successful pregnancy was achieved. In 1949, Ernest John Christopher Polge [8], biologist of English origin, accidentally discovered cryopreservation properties of 10 % glycerol in rooster sperm at −80 °C, reporting pregnancies with sperm that had been frozen at very low temperatures for over a year.

The application of cryopreservation of living cells and tissues revolutionized the fields of biotechnology, plant and animal improvement programs, and modern medicine at the time. The fact that prokaryotic and eukaryotic cells of the organisms could recover from temperatures up to almost 200° below the freezing point of water is considered a remarkable feat of endurance, as seen from our current understanding of the structural and molecular biology, but in most of these situations, the presence of cryoprotectants is indispensable. The term cryoprotectant is described as any additive that can be provided to the cells prior to freezing, in which survival obtained after thawing would not happen in the absence of this additive [4].

Glycerol, a poly-hydroxylated solute, with a high water solubility and low toxicity during short-term exposure to the living cells, interacts by hydrogen bonding with water and can penetrate through plasmatic membrane of many different cell types, but at a relatively slow pace.

Lovelock considered these properties and developed his theory of colligative action (property of a solution that depends only on its concentration) of cryoprotectants [4]. As per his theory, glycerol provides protection from freezing and thawing by acting as a colligative to reduce the high salt concentration that occurs during freezing. Solute depresses the equilibrium freezing point of a solution. By adding glycerol to a cryopreservation solution, the amount of water that freezes at any given temperature will be reduced. As a consequence, the final concentration of the salts in the remaining solution will also be reduced. He concluded that the colligative depression of the freezing point and concomitant reduction in the salt concentration explained the protective mechanism by which glycerol exerted its effect. This would prevent the critical concentration of salt to a harmful level, while the entire cell achieves cooling to achieve a vitreous state. The high viscosity of glycerol during the temperature drop also inhibits or delays the growth of ice crystals.

During the next two decades, a wide range of solutes (mainly alcohols, sugars, diols, and amides) were investigated as cryoprotectants, among them sucrose, 1,2 propanediol, ethanediol, and dimethyl sulfoxide (Me_2SO). Many other solutes of small molecular weight such as amino acids, including alanine, glycine, and proline; other sugars, including glucose, lactose, and ribose; and amides including formamide and acetamide were found to have cryoprotective effects but of low efficiency. In his opinion, Maximov [5] reported 56 solutes with cryoprotective activity, although many with very low efficacy.

In 1950, Bunge and Sherman [2] tried to use the same methods as Polge in human sperm reporting pregnancies of previously frozen

sperm. With these reports, the protective properties of glycerol and ethylene glycol against damage resulting from freezing of sperm were proven.

During 1953, Jerome K. Sherman, using frozen sperm at the University of Iowa, founded the first sperm bank [2].

Luyet in 1964, coined the term cryobiology, "cryo" meaning cold, "bios" meaning life, and "logos" meaning study, cryobiology is studies involving biological activity and structural changes of cells at subzero temperatures. Cryopreservation handles temperatures ranging from −80 to −196 °C. With the creation of the Society for Cryobiology in 1965, the name was changed from cryophylactic agents to cryoprotectants [1].

In the early 1970s, Whittingham et al. demonstrated that frozen mammalian embryos could survive temperatures as low as −269 °C temperatures and when thawed, survived and could be transferred into the uterus, resulting in pregnancies [9].

In humans, the first pregnancy reported in cryopreserved and thawed embryos, of eight cells, was in the year 1983 by Alan Trounson and Linda Mohr [10], which resulted in a spontaneous abortion at about 20 weeks gestation.

The difficulties associated with oocyte cryopreservation are related mostly to sensitivity and special structure these cells possess, exposure time to cryoprotectants, the concentration of cryoprotectants, equilibrium temperature, and the formation of intra- and extracellular ice that directly affects the viability of the cells [11].

The main disadvantages of the cryopreservation system by slow freezing of human embryos were the need for exponentially expensive material to achieve freezing and the time factor, as this process takes several hours to be carried out. The introduction of a technique that could be performed without the use of expensive equipment and took minutes to complete led to the creation of vitrification techniques [12].

Processes for a successful cryopreservation through vitrification of mammalian embryos and oocytes, including human, have been the subject of much research and protocols for many years. In 1985, Rall and Fahy first showed that a murine rodent embryo could be successfully cryopreserved by freezing using cryoprotectants, but the VS1 solution they used was highly toxic [1].

The way to decrease toxicity was decreasing the permeability of cells to toxic cryoprotectants, performing procedures in cold rooms at temperatures of 4 °C. Then the embryo is immersed in liquid nitrogen and gradually exposed to cryoprotectants for 50–60 min [1]. In 1985 Lassale et al. developed the use of propanediol and sucrose as cryoprotectants in protocols [12].

Immersing oocytes in liquid nitrogen directly is not enough to achieve successful vitrification. To achieve this we must avoid three problems: formation of ice crystals, effect of solutions and osmotic shock. These problems were identified, and gradually, various types of cryoprotectants had been developed. They can be divided into two groups: permeating and nonpermeating cryoprotectants [13].

Permeating cryoprotectants are small molecules that are readily permeable to the cell membrane. They form hydrogen bonds with water molecules and thereby prevent ice crystallization. At low concentrations of water, the temperature is lowest when mixed with it, but at high concentrations, it inhibits the formation of crystal, changing it to a vitrified solid state, wherein the water does not expand but only solidifies. In this way, ice crystals are avoided. These types of cryoprotectants are propylene glycol and propanediol [13]. They also offer protection to the cell against damage caused by solutions, because of their dilution of electrolytes.

The nonpermeating cryoprotectants remain extracellular. They function by extracting the water from the cell and creating a state of cellular dehydration. Used together, while nonpermeating cryoprotectants dehydrate the cell, permeating cryoprotectants prevent the creation of ice crystals in the cell. In the thawing cycle, nonpermeating cryoprotectants avoid osmotic shock, since the reduction of osmotic pressure in the extracellular area permits the exit of permeable cryoprotectants. If this does not happen, excessive water would enter the cell and may cause the rupture of the cell. The most common nonpermeating cryoprotectant used is sucrose, although other disaccharides can be used [13].

Vitrification commonly uses the mixture of permeating cryoprotectants at high concentrations such as DMSO and ethylene glycol, as well as sucrose, to dehydrate the embryo and to prepare it for cryopreservation, unlike slow freezing where the embryos are placed directly in liquid nitrogen [14].

Subsequently, ultrarapid vitrification systems were developed, which are tools that take a minute to vitrify volumes in sub-microliters with embryos. Several techniques have been developed with time, such as the open pulled straws system (OPS), vitrification on solid surface, vitrification with microdrops, Cryotop, nylon mesh system, CryoTip and Cryotec technologies, and other devices [14].

In 1998, Otoi et al. reported the first vitrification process using a simple straws system, obtaining better results than slow-freezing methods [15].

Subsequently in 1998, Vajta and M. Kuwayama et al. developed the system of OPS (Open pulled straws) to improve the method of vitrification in cattle. With the use of a minimal amount of cryoprotectants, they reported improved pregnancy rate. Being an open system, it was prone to dramatic changes in temperature in direct contact with liquid nitrogen and its use gradually declined [16].

Given these findings, a new CPS system (closed pulled straws) was invented with the same features of the open system with regard to the rapid temperature changes needed to vitrify, but modifying the temperature variation for a closed system [15].

Kuleshova et al. first reported the birth of a healthy baby, from an vitrified oocyte in 1999 after vitrification of 17 oocytes using 40 % ethylene glycol and 0.6 M/L of sucrose in a OPS [13].

In 2001, Mukaida et al. [17] reported that vitrification of human blastocysts was possible by suspension of embryos on a small nylon strap and placing directly in liquid nitrogen, known as cryoloop. The cryoloop, first described by Lane et al. in 1999, consists of a nylon strap 20 μm wide by 0.5–0.7 mm in diameter, mounted on a stainless steel pipette inserted into the lid of a cryovial. The use of this method reported a survival rate of 63 % after the 2-h devitrification process and also reported pregnancies and live birth of healthy babies.

The Hemi-straw device (Astro Medtec, Salzburg, Austria) is an embryo carrier that consists of a large gutter on which a small quantity of cryoprotectants (<1 μl) containing the cell is deposited. The Hemi-straw is subsequently inserted into a larger precooled 0.5 ml straw (CBS, Cryo Bio System, Grenoble, France) under liquid nitrogen. Prior to the commencement of the warming process, the Hemi-straw is pulled out of the larger straw under liquid nitrogen, and the tip of the Hemi-straw is immediately immersed into a Petri dish containing a sucrose solution. A rapid cooling rate of >20,000 °C/min is achieved by allowing direct contact of the biological material with LN2 [18].

The first extensive report series on vitrifications of human oocytes was published by Yoon et al. in 2003 where cryopreservation of 474 complex cumulus oocytes was achieved using 5.5 M ethylene glycol and 1.0 M sucrose as a cryoprotectant, reporting a survival of the oocytes to devitrify of 68.7 % and a fertilization rate of 71.7 %, an implantation rate of 6.4 %, and clinical pregnancy and live birth rate by transfer of 6/21 (21.4 %) [13].

In 2003 and 2004, Isachenko, Rahimi et al. reported a series of studies. They studied the morphology of the tissue in three groups: cryopreserved tissue by slow freezing using dimethyl sulfoxide (DMSO); cryopreserved tissue by vitrification and fresh tissue. It was observed that the morphology and hormone production was affected more in the two groups of cryopreservation than in the fresh [19].

The Cryotop system, a method created in 1999 (Kitazato Supply Co, Fujinomiya, Japan), by Masashigue Kuwayama [20], is an open vitrification system, where individual oocytes were picked up in an extremely small volume (<0.1 microl) of vitrification solution and placed on top of a very fine polypropylene strip (0.4 mm wide × 20 mm long × 0.1 mm thick) attached to a hard plastic handle specially constructed according to specifications by Kitazato. The droplet volume was estimated from the length of the fluid column within the pipette tip. As soon as the oocyte was placed onto the thin polypropylene strip of the Cryotop, it was immediately submerged vertically

into filtered LN2. Then the thin strip was covered with a hard plastic cover (3 cm long) on top of the Cryotop sheet to protect during storage in LN2 containers. For warming, the protective cover was removed from the Cryotop while it was still submerged in LN2, and the polypropylene strip of the Cryotop was immersed directly into the solution at 37 °C for 1 min. A cooling rate of 23,000 °C/min is obtained with the device. Minimum volume of solutions for vitrification increased the speed of cooling and thawing and decreased the amount of ice crystals formed during the freezing process. A mixture of ethylene glycol, DMSO (dimethyl sulfoxide), and sucrose was used as cryoprotectants. This method showed survival rates of 88 % after thawing.

The McGill Cryoleaf [18] is very similar to Cryotop but with a number of features designed to improve the loading and storage of cells. Safety during storage has been improved, as the cells are double protected from stress and contamination through a closed cover system but not hermetically sealed, leaving cells in direct contact with LN2. This device and the vitrification media have been developed by Dr. Chian and Prof. Tan at de McGill University, Montreal.

CryoTip [18] technology (Irvine Scientific) is a plastic straw container which can be sealed as a closed device to hold gametes or embryos in a specialized medium during cryopreservation procedures and subsequent long-term storage in a LN2 tank. CryoTip consists of a drawn plastic straw with an ultrafine tip and a protective metal cover sleeve. This device has been optimized as a closed system for cryopreservation procedures. For freezing, aseptically remove one CryoTip when ready to use. Aseptically attach the wide end of the CryoTip to an aspiration tool, such as a luer tip syringe, using the connector. When specimens are ready to load into the CryoTip, aseptically slide the metal cover sleeve carefully along the straw to expose the fine tip end. Gently load the specimens into the CryoTip by aspiration using the plunger on the syringe to control the uptake of medium and specimens. Heat seal the fine tip below the 1st mark, then slide the metal cover sleeve down over the fine tip to protect it, and plunge the sealed CryoTip into the LN2 reservoir. A cooling rate of 12,000 °C/min is obtained with this device [18]. This method is approved by the FDA; a minimum volume of solutions and cryoprotectants helps to eliminate the potential dangers of disease transmission and provides the possibility of cryopreservation in all phases of the oocytes and the development of preimplantation embryos in mammals [20].

Vitrification by microdrops was developed by Chia-Chieh Chang et al. in 2006. This technique is the creation of microdrops of 1–2 μl, containing a minimum amount of cryoprotectants during a short period of time, exposing the microdrops into liquid nitrogen to obtain a vitrified number. This process takes less than 2 min to complete, on a substrate such as paper or paraffin. It is a low-cost method, simple, and easy to operate [21].

The vitrification technique on a solid surface, developed in 2010 and reported by W. Xing et al., is a method which has very high survival rate in the preservation of oocytes and ovarian tissue. This technique uses a metal surface, previously frozen at −180 °C by partial immersion in liquid nitrogen, which serves as a base for the cooling of micro-droplets containing vitrification solutions, ovarian tissue, or embryos, which creates sufficient space for vitrification of tissues, maximizes freezing rates, and avoids the gas phase caused by bubbles of liquid nitrogen [22].

The Cryotissue is a special container for vitrification and cryopreservation of ovarian tissue, consisting of a metal strip attached to a plastic handle and a plastic cover; it can be used for transplantation of vitrified-thawed ovarian tissue giving high survival rate of oocytes. The first fertilization after vitrified ovarian tissue transplantation by using this device was achieved in March 2011. This device was developed by Noriko Kagawa Ph.D. (Advanced Medical Research Institute of Fertility at Kato Ladies Clinic) and has a cooling rate of −17,000 °C/min.

A new vitrification technique called Cryotech Vitrification, developed in 2012 by M. Kuwayama, offers a survival of 100 % after the thawing of bovine embryos. It allows the use of minimum volumes of cryoprotectants and allows the vitrification of oocytes and embryos at any stage of development. At the Mexican Infertility Institute

(IMI) in Guadalajara, Mexico, this device has been used since 2011, obtaining very high rates of survival after vitrification of oocytes and embryos, and this technique has been now used all over the world. Gandhi et al. reported the use of this technique in conjunction with minimal stimulation and reported excellent survival and pregnancy rates [23]. Kagalwala et al. compared this technique with Cryotop vitrification and reported very favorable results [24]. This technique is easy to use and safe in terms of risk of contamination.

Recent advances in technology have significantly improved vitrification efficiency of cryopreservation of oocytes in terms of survival, achievement of vitrified oocytes pregnancies, as well as live birth rates [16].

Certain challenges still remain associated with vitrification. These include development of less cytotoxic vitrification solutions, finding optimum cooling and warming rate using a safe vitrification device and most importantly, standardization of vitrification techniques in all laboratories. The effect of the duration of cryo-storage on the survival and development of cells also remains to be studied. Lastly, the health of infants born from vitrified oocytes need to be further evaluated in order to ensure safety of the vitrification technology [16].

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3 Vitrification of Oocytes: General Considerations

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Keywords
Cryopreservation • Fertility • Oocyte • Survival • Vitrification • Warming • Fertility preservation • Oocyte cryopreservation • Oocyte survival rate

Introduction

Cryopreservation of human cells is an integral part of assisted reproductive technology (ART). Sperm freezing is an established procedure and has been so for decades. As the rates of implantation with in vitro fertilization (IVF) improved, fewer embryos were being transferred owing to growing concerns of multiple pregnancies, and the cryopreservation of the supernumerary embryos was perceived as a necessity. The first successful report of human embryo freezing that generated a pregnancy was in 1983 [1]. Many rapid advances and improvements in the techniques of embryo freezing were made since, and a near consistent success rate was achieved with embryo freezing. Embryo freezing rapidly became established as a routine procedure in the ART lab.

Surprisingly though, enthusiasm for the cryopreservation of human oocytes remained conspicuous by its absence. Chen reported the first human birth using cryopreserved oocytes as early as in 1986 [2]. However, the failure to reproduce this early success became a major setback and the possibility of offering oocyte cryopreservation as a routine procedure remained remote. For a long time, it was considered asan experimental procedure at best. Significant resurgence of interest in the human egg freezing came about in recent times owing to its perceived potential to preserve fertility. Vitrification was applied to oocyte cryopreservation techniques, which produced more consistent and reproducible results. Kuleshova et al. reported the first birth from vitrified human oocytes [3]. Various studies followed, which showed superiority of vitrification over slow freezing in the post-thaw survival of vitrified oocytes [4, 5]. Vitrification was proposed as an alternative to conventional slow-freezing methods to avoid chilling injury

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and ice crystal formation. Recent studies have established that oocyte vitrification and subsequent warming are producing clinical results comparable to the results achieved by fresh cycles [6, 7].

Latest advances in cryobiology have led to a newer option or protocols available for the oocyte cryopreservation in terms of technique used, media composition, and various storage devices used for vitrification. This has resulted in an improved survival of cryopreserved oocytes. The American College of Obstetricians and Gynecologists (ACOG) endorses modern procedures to cryopreserve oocytes and no longer considers it experimental. The chapter aims at giving an overview of modern oocyte cryopreservation protocols and its indications and applications in current times.

Indications for Oocyte Freezing

- Loss of reproductive function in young women due to chemotherapy, radiation, and/or surgery for cancer
- Addressing the ethical and legal issues involved in embryo freezing
- Oocytes cryobanking for egg donation program
- In cases of premature menopause and then loss of ovarian function
- Women who want to delay childbearing
- Prevention of ovarian hyperstimulation syndrome
- Male factor infertility or inadequate seminal samples

Present trends show an increasing emphasis on health checkup among the general population, resulting in more aggressive cancer screening and earlier detection of cancers. This has meant earlier treatment and better survival rates. Advances made in the field of cancer treatments also have contributed to the increase in the population of cancer survivors. It is estimated that by 2015, one in every five cancer patients will survive and one in every 250 individuals will be a cancer survivor [8]. Chemotherapy and radiotherapy are known to decrease the fertility potential. The survival for these cancer patients comes at a cost of their fertility as chemotherapy has an adverse effect on ovarian function, and it is raising a new concern about their fertility [9]. Cryopreservation of ovarian tissue has been tried, but the potential risk of reintroducing cancer cells remains a matter to be seriously considered and counseled about. Embryos may be cryopreserved in patients with partners. The ethical and legal issues need to be discussed with the couple. Oocyte vitrification is the logical choice for the unpartnered and those with reservations with embryo freezing. Oocyte cryopreservation holds promise of fertility preservation for the cancer patients. Oocyte preservation should be recommended to those about to start gonadotoxic drugs or radiotherapy after adequate counseling. There has been no increase in the number of anomalies in children born from fertilizing the frozen oocytes demonstrated by a compilation of various studies. The pregnancy rates following oocyte cryopreservation although limited is however reassuring thereby making it a very attractive option for the cancer patients. A newer approach of cryopreserving immature oocytes from the luteal phase of the cycle has been recommended to prevent delay in the start of chemotherapy [10]. In vitro maturation (IVM) of the frozen oocytes prior to fertilization is then performed, while certain investigators have recommended IVM prior to the cryopreservation.

An application of oocyte vitrification likely to gain immense popularity is the establishment of donor oocyte banks for egg donation program. The oocyte donation practiced today involves the use of fresh oocytes, which requires the synchronization of periods required between the donor and the recipient. This either restricts the choice of donors for the recipient or causes delays in the cycle for clients who are very particular about the donor choice. These delays and restrictions increase the cost of treatment. Occasionally, unexpected events in a donor's life have led her to being unable to donate at a particular time, causing immense frustrations and loss of money for the recipient. Vitrifying oocytes and creating an oocyte bank can overcome these restrictions, much along the same lines as a sperm bank. The recipient is assured of a donor of her choice at

any time that she plans her cycle while the donor is also benefitted since she can donate eggs at her convenience. Donor oocyte banking potentially offers more choices for donor selection and more flexibility in the timing of treatment cycles and eventually cost reduction.

Assisted reproduction involves controlled ovarian stimulation and creating many embryos. As the rate of implantation increased, the number of embryos transferred started decreasing amid growing concerns of multiple pregnancies and associated complications. Cryopreservation of supernumerary embryos while a logical option raised ethical and legal concerns. There were clients with ethical and moral reservation with cryopreserving embryos, while certain countries like Germany and Italy continued to remain reluctant to endorse embryo freezing and even outright forbidding it. Legally too, there have been conflicts arising owing to a lack of definition or even a clear consensus with regard to the status of the embryos. There were some assigning the embryos the status of a human being with legal rights, while others consigning the embryos a status of an unborn child with no legal rights.

These dilemmas are of particular concerns in present times where transfer of fewer embryos is the trend and certain medical situations which demand the cryopreservation of all embryos as in prevention of OHSS a potentially life-threatening situation. Cryopreservation of oocyte addresses many of the ethical and legal concerns, and a well-established oocyte preservation program will provide viable option in many of these situations.

Every woman is born with a finite oocyte reservoir, and with age, there is an increased depletion in numbers also termed as "gamete exhaustion" along with the deterioration of oocyte quality. In women with a family history of early menopause, oocyte depletion is much faster than usual. Oocyte freezing can be an effective tool to preserve their eggs.

Certain genetic conditions like Turner mosaic are associated with premature menopause, and oocyte cryopreservation has been suggested in this group; however, the risk of transmission of the congenital anomalies and the safety and efficacy of the process need adequate consideration. For women who wish to delay childbearing, oocyte cryopreservation may be considered an option. However, the data for this group is very minimal with little data available of its efficacy. Promoting the use of oocyte cryopreservation in this group may give false hopes and may encourage delaying childbirth in women. The counseling in this group needs to address each of these issues and the potential risk of child bearing at a later age.

Challenges Associated with Oocyte Vitrification

Oocyte cryopreservation is technically more challenging than embryo cryopreservation. This is because the oocyte is a very sensitive cell. Shape, size and cell number are important factors affecting survival during cryopreservation and explain why it has taken so long to introduce oocyte cryopreservation even though the first embryo was cryopreserved 40 years ago.

Factors Affecting Oocyte Survival

(a) *Spherical shape*: Perfect sphere of the oocyte slows down permeation and equal distribution of cryoprotectant in the oocyte. This continuous concentration gradient results in longer exposure of oocyte to cryoprotectants, which may lead to toxic damage in one part of the oocyte offering less than optimal protection in the other. Therefore, the changes in the shape at the equilibration step due to osmotic effect may provide some type of benefits, but the cytoskeleton damage of the oocyte may also be triggered [11].

(b) *Size*: Size is a very important parameter in cryobiology. The larger the cell, the more difficult to cryopreserve it. Oocytes are the largest cell of the human body. Size of the oocyte widely affects crystal formation and the slow dilution or accumulation of toxic cryoprotectants, thus increasing the challenge of survival.

Fig. 3.1 Principle of vitrification

Successful vitrification = $\frac{\text{Cooling and warming rate} \times \text{viscosity}}{\text{Volume}}$

(c) *Low cell number*: Since oocyte is a single-cell structure, there is no backup to regenerate from serious injuries as compared to multicellular embryo, which can survive up to 50 % loss of its cells [11].
(d) *Osmotic shock:* The osmotic shock that can occur during dilution may result in extensive swelling, rupture of the membrane, and immediate death of the oocyte.
(e) *Cryoinjuries*: The oocyte is highly susceptible to cryoinjuries to the cytoplasmic content and the nuclear spindle [12]. Chilling injury occurs at high temperature and induces irreversible damage to the cytosolic content, membrane, and zona pellucida. Hardening of the zona due to premature cortical granule release may result in decreased fertilization rate. Therefore, a very careful approach has to be applied for oocyte freezing.

Due to these varied difficulties associated with oocyte vitrification, for many years, oocyte vitrification was considered experimental, and, therefore, embryo cryopreservation was the only established option for fertility preservation in female cancer patients [13].

Vitrification

Vitrification is a process of cryopreservation which involves exposure of oocyte to high concentration of cryoprotectants and ultrarapid cooling to solidify the cell into glass-like state without the formation of ice crystals. In the vitrification process, cooling rate in the range of 2,500–30,000 °C/min or greater is used, which results in the water of the cells to transform from the liquid phase to a glassy vitrified state. During the vitrification process, an oocyte is placed in a small volume of the vitrification medium and is then cooled at an extremely rapid rate. High osmolarity of the vitrification medium allows rapid dehydration of the cells, and immersion into liquid nitrogen solidifies the cells. This ultrarapid freezing eliminates the formation of damaging ice crystals in the cells. Though the high concentrations of cryoprotectants are toxic to oocytes, its toxicity can be minimized by increasing the cooling rate and also to prevent chilling damage [14]. For successful vitrification, a very high cooling and warming rates are required to reduce the concentration and toxicity of cryoprotectants (Fig. 3.1).

Time Schedule for Vitrification of Oocytes and Fertilization

Oocytes can be vitrified 2–4 h after the oocyte retrieval. Oocytes are stripped of cumulus cells by hyaluronic acid treatment. By stripping the cumulus cells from the oocytes, maturity of the oocyte can be assessed. A study by Chian et al. on bovine oocyte vitrification has reported that cells without cumulus had a higher survival rate and superior embryonic developmental capacity after warming as compared to oocytes vitrified with cumulus cells [15].

The meiotic spindle of the oocyte is very sensitive to cryoprotectants and low temperature [16, 17]. Oocytes analyzed immediately after thawing displayed severe disorganization or disappearance of spindles [18]. Although the spindle transiently disappears immediately after thawing, it reorganizes/reforms with good morphology after 1–3 h of in vitro culture at 37–38 °C in the majority of mature oocytes [19]. Inseminating oocytes soon after thawing, when there is serious spindle disorganization, adversely affects fertilization outcome and growth of embryos [18]. The optimum time for intracytoplasmic sperm injection (ICSI) for human oocytes is between 37 and 41 h post human chorionic gonadotropin (hCG). The time interval between oocyte thawing and ICSI is crucial for normal fertilization and subsequent development. Optimizing the time of ICSI, which involves giving adequate time for spindle recovery after warming,

Table 3.1 Types of cryodevices

Device	Vitrification system	Description	Merit
Electron microscope copper grid [20]	Open	Small copper disc with a fine mesh	It improves heat conduction to oocyte cytoplasm thereby prevents osmotic damage cause by increased concentration of vitrification solution
Open pulled straw [21]	Semi-closed	Pulled straw inner tip diameter approx. 0.8 mm	Renders high cooling and warming rate (>20,000 ° C/min)
			Easy loading process, "autoloading" by the capillary forces of OPS
Closed pulled straw [22, 23]	Closed	Pulled straw	It gives high survival rate and preserves spindle fiber
Cryoloop [24, 25]	Open	A thin nylon loop (0.5–0.7 mm in diameter)	Easy to perform and demonstrates stages of development of oocytes and day 3 embryos. Lower concentration of cryoprotectant is used
Cryotip [26]	Closed	Finely pulled plastic straw (250 μm inner diameter)	This system eliminates the potential for embryo contamination during cryopreservation and storage
Cryoleaf [26]	Open		Easy to handle and efficient storage of oocytes. Vitrified cells are protected from mechanical stress and contamination by a closed cover system
Cryotop [27]	Open	A fine polypropylene strip attached to a plastic handle and equipped with a cover straw	Cryotop technology is the minimal volume approach for vitrification. Easy to load and store oocytes or embryos
Cryotec [28]	Open and closed	A strip attached to a plastic handle and equipped with a cover straw	It is a relatively newer approach in minimal volume methods and is a very efficient technique in terms of survival embryo quality, implantation rates, and clinical pregnancy

while at the same time avoiding undue delay, which could result in oocyte aging related damage, is essential for the success of the program. Therefore, considering competing aspects of oocyte aging and spindle recovery is essential for a successful oocyte cryopreservation program. Hence, oocyte recovery can be performed at 34 h post hCG. Vitrification of oocytes can be performed at 2 h after oocyte recovery, and ICSI is being performed at 3 h post thaw, adding up to 39 h post hCG.

Vitrification Devices

Various open and closed system cryodevices are available today with their advantages and drawbacks as described in Table 3.1. Most vitrification protocols use an open system in which oocytes are directly exposed to liquid nitrogen to maximize cooling and minimize formation of ice crystals. Closed system device involves indirect contact with liquid nitrogen. With the use of open vitrification methods, theoretic infectious disease transmission is one of the concerns as the oocyte is directly exposed to the contaminated liquid nitrogen. Studies have suggested that infectious pathogens can survive in liquid nitrogen [29, 30]. For safety issues, various methods to sterilize the liquid nitrogen have been developed [31]. The hypothetical risk of contamination may also be possible in closed system during the warming procedure [31, 32]. Although the infectious disease transmission with the use of open vitrification system was of great concern, but there are studies which suggest that it was never observed in reproductive tissues [33].

Current advances in cryopreservation have resulted in devices that hold minimal amount of

cryoprotectant media containing the specimen, referred as minimum volume approaches. High cooling and warming rates along with the minimal volume methods help to avoid chilling and fracture injury. Therefore, scientists have made great efforts to find new methods using minimum volume techniques to increase the thermal change (Table 3.2) [20–28].

Vitrification: Cryotech Method (Cryotech, Japan)

Dr. Masashige Kuwayama has introduced various novel vitrification techniques. His minimal volume vitrification techniques are used worldwide producing excellent results. Cryotech vitrification "a minimal volume approach" is the latest and innovative method of oocyte and embryo cryopreservation introduced by Dr. Kuwayama. It is a safe and extremely efficient method of vitrification. Protocol of cryotech vitrification has been optimized to preserve oocytes and embryos of any developmental stage. With major improvements in the solutions and tools, cryotech vitrification has shown excellent results in terms of oocyte survival, fertilization rate, and pregnancy rate.

Table 3.2 Minimum volume methods

Vitrification method	Volume (ul)	Cooling rate (°C/min)	Warming rate (°C/min)
OPS [21]	1–2	16,700	13,900
Cryoloop [25]	<1	20,000	
Cryotop [26]	<0.1	23,000	42,100
Cryotip [26]	1	12,000	24,000

Cryotech Method of Vitrification

Cryotech vitrification includes equilibration step where oocytes are placed in equilibration solution and exposed to the lower strength of the cryoprotectants. Oocytes are incubated until they have recovered from osmotic shock. The equilibration results in dehydration of the cell and its permeation with cryoprotectant, which is visible by changes in the shape of an oocyte. The shape of an oocyte changes as it undergoes transient shrinkage with the loss of water and then regains its original shape as the cryoprotectant permeates into the cells (Figs. 3.2 and 3.3).

After the completion of the equilibration step, oocytes are then placed in vitrification medium which is a solution having higher strength of cryoprotectants. The exposure time of oocytes in the vitrification medium is very short, approx 60 s. This is followed by oocyte loading on cryotec (filmstrip), a carrier device, and direct plunging in liquid nitrogen. To ensure maximum cooling rate (23,000 °C/min), cryotec should be submerged in liquid nitrogen with continuous vertical movements. Cryotec is then covered with the cap still under liquid nitrogen. This helps in avoiding mechanical damage during storage (Figs. 3.4, 3.5, 3.6, 3.7, and 3.8).

Storage

After the vitrification process, the vitrified oocytes in cryotec are stored in liquid nitrogen until it is to be warmed and fertilized by a sperm. Vitrification thus involves a technique where the oocytes can be vitrified without damage and can be safely preserved for future use.

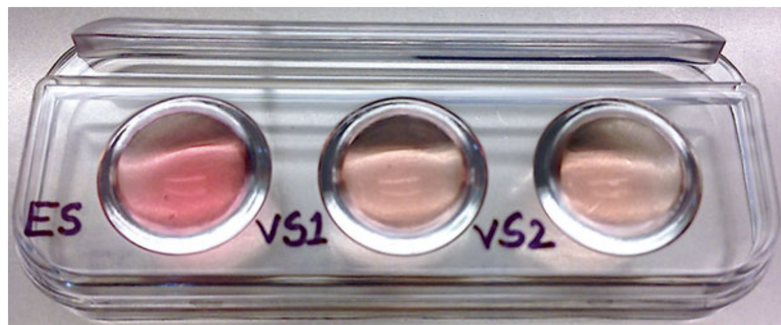

Fig. 3.2 Cryotec vitrification plate with solutions *ES* - Equilibration Solution, *VS1* - Vitrification Solution 1, *VS2* - Vitrification Solution 2

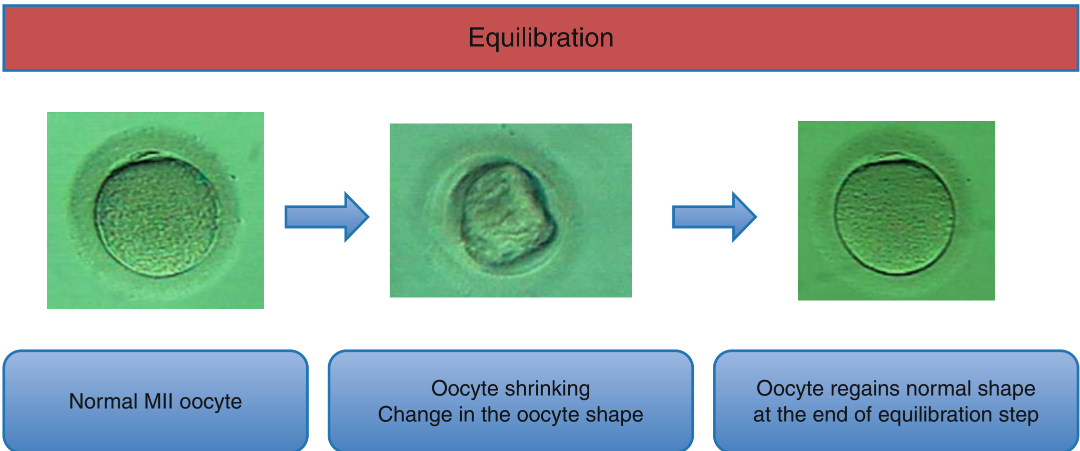

Fig. 3.3 Equilibration step

Fig. 3.4 Cryotec vitrification plate with cryotec placed in the groove

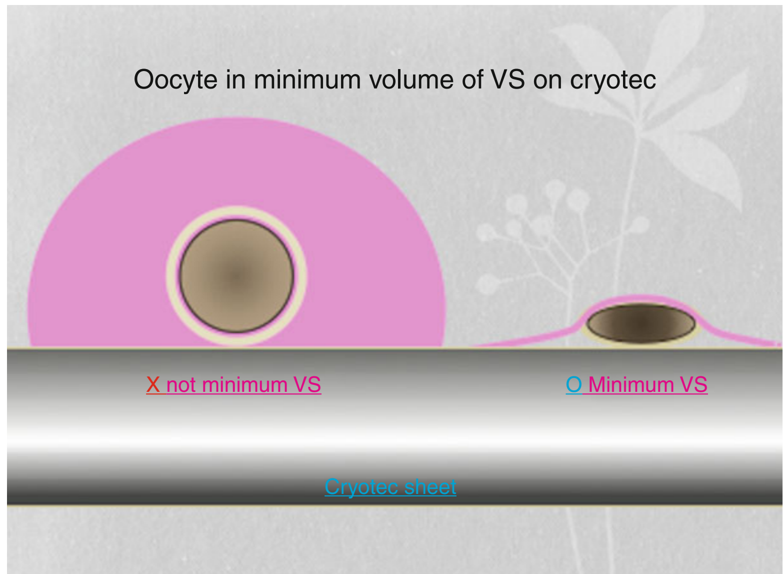

Fig. 3.5 Loading the oocyte with minimum volume
ES - Equilibration Solution,
VS - Vitrification Solution 1,
VS - Vitrification Solution 2

Fig. 3.6 Loading the oocytes onto the cryotec

Fig. 3.7 Dipping the loaded cryotec into liquid nitrogen

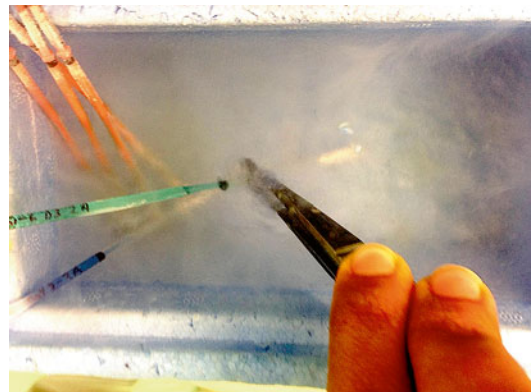

Fig. 3.8 Covering the loaded cryotec with the sleeve under liquid nitrogen

Warming

Warming is the reverse of the entire process of vitrification. Warming involves the immediate transfer of the vitrified oocytes to a pre-warmed (37 °C) environment. In warming, vitrified oocytes are exposed to hypotonic solutions of decreasing strengths of warming media in a stepwise manner to remove the cryoprotectant. Oocyte undergoes transient volume expansion during the removal of the cryoprotectant and gradually rehydrates.

Cryotech Method of Warming

For warming, the protective cover of the cryotec is removed and the film-strip holding the vitrified oocyte is quickly immersed into the warming solution which is maintained at 37 °C. This results in achieving extremely high warming rate (42,000 °C/min). After the incubation period of 1 min, oocyte is placed in the dilution solution for 3 min. The oocyte is then washed in washing solution for 5 min. Survival judgment is made at the end of this step depending on the recovery of

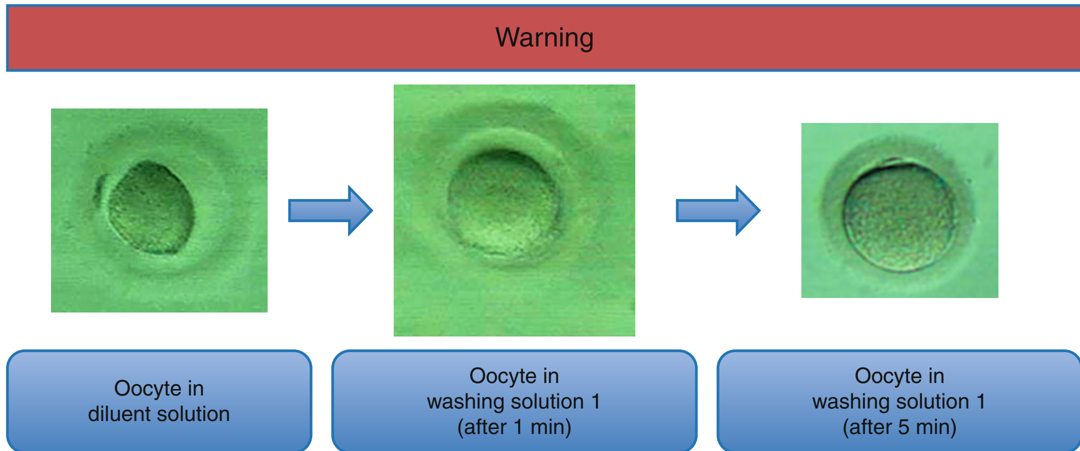

Fig. 3.9 Changes in oocyte shape during the warming process

the shrunken oocyte. The oocyte is again washed in washing medium and then placed in the culture medium (Fig. 3.9).

After warming, the oocytes can be fertilized using intracytoplasmic sperm injection (ICSI), as zona hardening is reported after warming [34].

Advantages of Cryotech Vitrification Technique

The Cryotech method has various advantages as compared to other techniques, in terms of solution, tools, and technique used.

1. *Solutions*
   - There is no added serum and synthetic serum supplements in vitrification solution. Hence, risk of serum-derived virus contamination is avoided.
   - Vitrification solution is completely chemically defined and is stable for 1 year at 4–8 °C and 3 months at room temperature.
   - It contains hydroxylpropyl cellulose (HPC), which has optimal high viscosity providing better resistance to damage during cooling, storage, and warming.
   - Sucrose is replaced with trehalose, thus reducing endotoxicity due to sucrose.
2. *Tools*
   - The plate used for vitrification has a special holder for the cryotec. Thus, the focus remains the same while washing the oocytes in vitrification solution and placing them on the cryotec.
   - The carrier device (cryotec) is a filmstrip attached to a plastic handle, equipped with a cap to cover the filmstrip for safe handling and storage.
   - There is no blind space in wells of vitrification and warming plates, so the chances of losing the oocyte is very rare.

Clinical Outcomes

In various randomized controlled trials where the clinical outcome of cryopreserved and fresh oocytes was compared by using the similar vitrification protocol, it was observed that there is no significant difference in fertilization and pregnancy rates in cycles with fresh oocytes and vitrified oocytes [1, 2]. It was further supported by a recent meta-analysis of five randomized controlled trials from 2011 where the efficacy of oocyte vitrification was assessed. Parameters like survival rate, fertilization rate, embryo development, and pregnancy rate were assessed after comparing the two methods of vitrification and slow freezing. There was no difference found in pregnancy rate, top-quality embryo, embryo cleavage, and fertilization rate between the vitrified and the fresh oocyte groups. However, the

oocyte survival rate, fertilization rate, and cleavage rate were found to be higher in vitrified oocytes when compared to slow frozen oocytes. Also the top-quality embryos formed were higher in vitrified oocytes as compared to the slow frozen oocytes [35]. These studies suggest that vitrification is a simple and effective method for the cryopreservation of oocytes [36].

In view of the results reported across the globe, the American Society for Reproductive Medicine (ASRM) in 2012 declared that oocyte vitrification is no longer considered experimental. The report entitled "Mature Oocyte Cryopreservation" replaced a report released in 2008 that had stated that the technique was experimental and should only be offered in that context [35]. The report examined nearly 1,000 published papers and found sufficient evidence to "demonstrate acceptable success rates in young highly selected populations." The report does urge caution, however. The Committee points out that the age of the woman at the time of egg freezing is a very important factor.

Our Experience with Oocyte Vitrification

A comparative study to evaluate the outcome of oocyte vitrification using the Cryotech method, observed in an egg donation program with fresh versus frozen oocyte, was done at Rotunda – The Center for Human Reproduction. The data was evaluated over a period from January 2011 to December 2011. A total of 1,210 oocytes were obtained from 112 oocyte donors. The analysis included a total of 1,029 mature oocytes, 485 oocytes were used in fresh cycle, and the remaining 544 oocytes were vitrified using the Cryotech method. The vitrified oocytes were subsequently warmed using the Cryotech warming method. The warmed oocytes were fertilized by ICSI, and the embryos thus created were transferred into the recipients. A total of 171 embryo transfer cycles were performed on day 3 of embryo development. The parameters, which were assessed and compared between the two groups, included survival rate, fertilization rate, cleavage rate, embryo development, and the clinical pregnancy rates (Tables 3.3 and 3.4).

Table 3.3 Comparative data showing survival, fertilization, and cleavage rates between fresh and frozen–thawed human oocytes

	Fresh oocytes	Frozen–thawed oocytes	*P*-value
No. of oocytes	485	544	
Survival rate (%)	NA	94.5	
Fertilization rate (%)	83.0	80.9	NS
Cleavage rate (%)	96.5	94.4	NS

Table 3.4 Comparative data showing clinical outcome between fresh and frozen–thawed human oocytes

	Fresh oocytes	Frozen–thawed oocytes	*P*-value
No. of cycles	80	91	
Pregnancy rate (%)	56.3	54.9	NS
Implantation rate (%)	28.0	31.1	NS
Biochemical pregnancy rate (%)	8.8	8.0	NS
Missed abortion rate (%)	11.1	12.0	NS
Live birth rate (%)	45.0	45.1	NS

With these encouraging results, it is proved that Cryotech vitrification is an effective method that preserves the potential of vitrified oocytes to fertilize and further develop, which is similar to fresh oocytes. Excellent clinical outcome indicates that this technology can be applied successfully for the oocyte vitrification in various indications and egg donation programs. Thus, Cryotech vitrification holds a great promise in infertility management by offering a successful oocyte vitrification program.

Important Tips to Be Followed for Cryotech Vitrification

1. It is recommended to use a pipette with an inner diameter of 140–150 μm for the oocyte.
2. Close adherence to the recommended protocol of the manufacturer is absolutely essential to achieve optimal results.
3. The vitrification and warming process is to be done at RT.
4. The vitrification solutions must be tempered to 25–27 °C at least 1 h before using.

5. The plate used for warming and warming solution vial should be incubated at 37 °C at least 3 h before use (overnight incubation recommended).
6. Dilution and washing must be tempered to 25–27 °C at least 1 h before using.
7. Oocytes can be vitrified ideally 1 h after denuding.

Safety of Vitrification

There have been many concerns regarding the safety of vitrification. With vitrification techniques being used in thousands of labs all over the world, it is very essential to establish the safety of the method. IVF has potential risk. Not just oocyte vitrification, any kind of assisted reproductive technique has some potential risk. There are many risks involved like proteomic, metabolic, epigenetic.

A study on the metabolomic profile of embryos developed from fresh and vitrified oocytes has suggested that embryonic metabolomic profile is not disturbed by oocyte vitrification [37]. The influence of vitrification on the oocyte proteome of in vitro-grown germinal vesicle (GV) as well as metaphase II (MII) oocytes was assessed in a study on mouse oocytes. Transient changes in mitochondrial activity by vitrification occur at the pre-antral stage, but proteome of in vitro-grown and matured oocyte is not affected [38]. Oocyte proteome and developmental potential may get affected [39] because of changes in cellular integrity and protein alterations with the use of high concentration of cryoprotectant in an animal model [40, 41].

In a study where messenger RNA (mRNA) contents in MII oocytes after slow-freezing/rapid-thawing and vitrification/warming protocols was compared with the fresh MII oocytes, it was found that mRNA abundance may decrease in both type of cryopreservation methods which may result in molecular injury. However, the mRNA content level in vitrified oocyte remains sufficient to sustain biological function [42]. In another study gene expression profile of human metaphase II (MII) oocytes in both slow freezing and vitrification suggests that both methods modify gene expression profile of MII oocyte [43]. Animal studies indicate that ART techniques are associated with epigenetic alternations, like DNA methylation, but it is not recommended to infer such findings to human embryology [44].

Various publications have shown that there is no disturbance of meiotic spindle and abnormal chromosomes from previously frozen oocytes [45, 46]. Recovery of the meiotic spindle occurs after cryopreservation in both conventional and vitrification technology [47–49]. Thus, it is suggested that there is no increase risks of disturbances in spindle formation or chromosome segregation with vitrification of oocytes [50]. Chromosomal abnormalities are generally associated with age. Various data has revealed that there is abnormal meiotic spindle in older women in terms of chromosomal alignment and the microtubule matrix that comprise the meiotic spindle. In younger population of females, a well-organized meiotic spindle with fully aligned chromosome is observed. Such study has shown that high prevalence of aneuploidy is observed in older females as their meiotic spindle is altered with age [51, 52].

In a study conducted to check the neonatal outcomes of babies born using vitrified oocytes, a total of 58 reports were reviewed from 1996 to 2008. Of the total 936 newborns, 1.3 % had birth anomalies. No difference was noted in the birth defect rates when compared with naturally conceived babies [53]. A similar study has revealed no difference in birth weight or congenital anomalies among those born from vitrified oocytes as compared to children conceived after fresh IVF [54, 55]. A successful live birth has been reported from vitrified oocytes after 5 years of cryopreservation [56]. These findings suggest that there are no reported increase in miscarriages, chromosome abnormalities, and birth defect in the infants born from vitrified oocytes. With many recent studies now reporting similar findings, vitrification procedures have certainly become mainstream and it increases our confidence in oocyte vitrification. Although these data are reassuring, long-term health of children born from vitrified oocytes should be assessed mainly the epigenetic

effects of the treatment. Intracytoplasmic sperm injection (ICSI) used to fertilize the vitrified warmed oocyte may be associated with epigenetic effects [57].

In cancer treatment, ovarian reserves are compromised as chemotherapy, radiation, and surgery affect the reproductive system; oocyte cryopreservation offers a chance to preserve the reproductive capability of a female. Fertility preservation approaches should be discussed before the start of the treatment. If a woman is diagnosed with cancer at a young age, it is advisable to freeze the oocyte for use in the future. For girls who have not reached puberty, ovarian tissue can also be frozen. With the help of IVF or in vitro fertilization of cryopreserved oocytes and subsequent embryo transfer, many cancer survivors can have children. Oocytes can also be preserved after the treatment of cancer even though these are considered pretreatment options as in some cases viable eggs may be present after cancer treatment. In a study, no unusual cancer risk has been identified in the offspring of cancer survivors except in families with genetic cancer syndromes like inherited retinoblastoma [58, 59]. Thus, with the use of oocyte cryopreservation, reproductive capacity of a female can be prolonged and protected.

Conclusion

Oocyte cryopreservation by vitrification is emerging as a very useful tool in ART clinics with many applications. Children born from cryopreserved oocytes have not shown any increase in chromosomal abnormalities, birth defects, or developmental deficits. The success of oocyte vitrification has improved with advances in the vitrification technology, and the data obtained from various studies are reassuring. Current data supports the idea of an efficient oocyte vitrification program in all ART laboratories.

Technology advances rapidly in the area of assisted reproduction, considering the demand for assurances of success, and we can expect that oocyte cryopreservation techniques will continue to become more sophisticated and easier for embryologists to use. The scope of its use and application is only likely to increase in the times to come. Many more interesting and fantastic dimension of research is also currently being done on animal gametes, where newer techniques are under study and will no doubt have some applicability for human endeavors as well, somewhere in the distant future.

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Sperm Vitrification

4

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Keywords
Vitrification • Sperm cryopreservation • Human spermatozoa • Slow freezing • Cryodamage

Introduction

In the context of cryobiology of reproductive cells and tissues, sperm cryopreservation has the longest history. Due to the robust nature of the male gamete, it can be indefinitely stored in liquid nitrogen without compromising its ability to be used for the treatment of infertility. Cryopreservation is a process where we can preserve cells and tissues by cooling them to low sub-zero temperatures such that it can be revived and restored to the same living state as before it was stored. From the earliest reported experiments of freezing of human spermatozoa [1–3], there have been many improvements in the methodology of human sperm cryopreservation [1–3]. Sperm cryopreservation can be done using rapid- and slow-cooling (programmable or non-programmable) methods. Slow freezing involves gradual acclimatization of the sperms to a low temperature, whereas in vitrification the sperms are instantly subjected to a very low temperature.

Semen cryopreservation is beneficial before therapy for malignant diseases, vasectomy, or surgical infertility treatments, to store donor and partner spermatozoa before assisted reproduction treatments. Despite its usefulness, cryopreservation may lead to deleterious changes of sperm structure and function. It is well accepted that the freeze–thaw process affects the fertility potential of spermatozoa. Cryopreservation results in decreased sperm motility [4], alteration in cell membrane fluidity [5, 6], and changes in acrosome integrity [7] and induces apoptosis [8]. Sperm cryopreservation is an important component of fertility management and much of its successful application seems to affect the reproductive outcome of assisted reproduction technologies (ART).

Fundamentals of Cryobiology

Cryopreservation involves cooling of cells and tissues to a temperature where all the metabolic processes are suspended. Thus, the technology of cryopreservation enables us to pause the biological clock by hindering its growth.

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Effective cryopreservation of cells and tissues comprises of the following major steps:

(a) Adding a cryoprotective agent (CPA) to the cells
(b) Subjecting the cell suspension to low temperature, optimized to minimize intracellular ice formation
(c) After thawing, removing the CPA from the cells

CPAs can be classified into two groups, low-molecular-weight penetrating solutes like glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, or propylene glycol that pass through the plasma membrane and high-molecular-weight non-permeating polymers like sucrose, polyvinylpyrrolidone (PVP), hydroxyl ethyl starch, or dextran that do not pass through the plasma membrane [9–11]. Both penetrating and non-penetrating CPAs reduce the extent of ice crystal formation during cryopreservation by reducing the concentration of intracellular water [12]. Addition of the hypertonic cryoprotective agent to the isotonic cell suspension results in the distinctive "shrink–swell" behavior. Osmotic efflux of water is faster than the rate at which the cryoprotectant is diffused into the cell; hence, the cell dehydrates and shrinks. As the cell dehydrates, the rate of water efflux reduces and the rate of CPA diffusion increases causing the cell to swell. Due to the osmotic pressure difference created by CPA diffusion, the water reenters the cell to maintain chemical equilibrium. In spite of its benefits, CPAs are known to play an important role in causing cryoinjury to the cells due to osmotic stresses [9, 11]. During freezing if the cells are cooled too quickly and water is not lost rapidly, it leads to intracellular ice formation which is detrimental to the cells [9]. On the other hand, if cooling is very slow, the cells will lose water rapidly and dehydrate without intracellular freezing due to insufficient intracellular solutes [9]. Hence, a cooling rate "too high" or "too low" can cause cell death.

A cell that has survived cooling to low subzero temperatures still faces challenges during warming and thawing, which can exert effects on survival comparable to those of cooling. During the process of thawing and removal of CPA, stages opposite to that of freezing are observed, as the cells swell first due to osmotic influx of water and then as the CPA and water leave the cell, they return to their initial isotonic volume [11, 12].

There are two methods of cryopreservation, viz., slow rate freezing and vitrification. The basic objective of both these methods is the same, i.e., to protect cells from cooling effects (chilling injury), intracellular ice formation, dehydration, and toxic effects at both high and low temperatures.

Conventional Slow Freezing and Thawing

Slow cooling method involves a brief pre-equilibration of cells in cryoprotectant solutions followed by slow, gradual, controlled cooling at rates optimized for the type of cells being cryopreserved. The use of programmable or nonprogrammable "slow" (conventional) freezing allows preserving relatively large volume of diluted ejaculate or prepared spermatozoa with sufficiently high-quality post-thaw characteristics. It does also provide acceptable protection against membrane changes and destabilization induced by cryopreservation. To avoid the lethal intracellular ice formation, the cryoprotective solution contains buffers, carbohydrates, salts, egg yolk, and antibiotics. Permeable cryoprotectant glycerol is widely used in sperm vitrification.

The slow-freezing technique proposed by Behrman and Sawada [13] consists of progressive sperm cooling over a period of 2–4 h in two or three steps, either manually or automatically using a semi-programmable freezer. The manual method is performed by simultaneously decreasing the temperature of the semen specimen while adding a cryoprotectant in a stepwise manner and later plunging the specimen into liquid nitrogen. It has been shown that the optimal initial cooling rate of the specimen from room temperature to 5 °C is 0.5–1 °C/min. The sample is then frozen from 5 to −80 °C at a rate of 1–10 °C/min. The

specimen is then plunged into liquid nitrogen at −196 °C. In spite of the reports showing successful sperm freezing with manual techniques, the reproducibility of this procedure poses some problems. For this reason, programmable freezers are preferred which use the software data logging to obtain cooling from 20 to −80 °C at the rate of 1.5 °C/min and then at 6 °C/min. At completion of freezing, the straws are removed and stored into liquid nitrogen at −196 °C. This takes about 40 min. Programs are simple to use and allow for a cooling combination that does not require continuous operator intervention and have been used to increase the reproducibility of the freezing operations.

Conventional slow freezing, either manual or automated, can cause extensive chemical–physical damage to the sperm.

Detrimental Effects of Cryopreservation on Sperm Integrity

Compared with other cell types, spermatozoa seem to be less sensitive to cryopreservation damage because of the high fluidity of the membrane and the low water content (about 50 %). Despite this, cryopreservation may lead to deleterious changes of sperm structure and function.

Motility

Sperm motility is more susceptible to cryodamage and consequently affects its fertilizing capacity. Post-thaw samples show almost 50 % reduction in motility [14]. This functional impairment is due to structural damage of flagella, plasma membrane, acrosome, and mitochondria. Some studies have revealed that the freeze–thaw process may trigger the oxidative stress which leads to low motility [15, 16].

Viability

The success and usefulness of cryopreservation is measured by the number viable sperms. Viability of the sperms reduces to some extent after the freeze–thaw procedure. Apoptotic cascade may be triggered by the freezing process which results in nonviability of some sperms [17].

Morphology

Cryodamage leads to morphological impairment such as vacuolation in the cytoplasm, amorphous sperm head, midpiece anomalies, and loss of membrane integrity. Some studies have revealed that the freeze–thaw process leads to numerous alterations in the cell membrane which may affect the fertilization ability of the sperm [18].

Acrosome Activity

Decreased acrosome activity and reduction in intact acrosomal cap results in low sperm penetration in cervical mucus [19].

Mechanisms and Manifestations of Cryodamage in Spermatozoa

Human sperm shows a specific cryophysical behavior, and cryodamage affects different levels and functions of the cell. Cryopreservation causes a wide range of damage affecting sperm at molecular and functional level.

1. *Effect of cryoprotectants*
   Cryoprotective agents are used in cryopreservation to improve cryosurvival rate. CPAs lower water freezing point and prevent ice crystal formation to avoid structural damage. CPAs can be toxic if used in high concentrations, as cryoprotectant induces osmotic changes. Higher glycerol concentrations have toxic effects on mitochondria [20]. Inappropriate concentrations of CPAs and cooling rate can cause intracellular and extracellular ice formation that can be sublethal to the spermatozoa. If the cell undergoes excessive dehydration, membrane damage and denaturation of proteins due to shift in pH may occur.
2. *Intracellular ice formation*
   Intracellular ice formation has been often attributed to be the cause of cellular damages that human spermatozoa encounter at rapid rates of cooling. However, it has been demonstrated by Morris that cell damage to human spermatozoa, at cooling rates of up to 3,000 °C/min, is not caused by intracellular ice formation [21].

3. *Extracellular ice formation*
   Rapid cooling rate results in extracellular ice formation in the cryosolution surrounding the spermatozoa causing cell damage. Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing. It is believed that either the crystallization during freezing or recrystallization during thawing could be a major cause of sperm cryoinjury. The osmotic efflux of water by hypertonic cryoprotectants and raised osmolality as a result of extracellular ice formation leads to cellular dehydration, changes in intracellular electrolyte concentrations, and disruption of sperm membrane permeability [22].
4. *Osmotic imbalance*
   With their high proportion of membrane lipids, sperm in particular are sensitive to osmotic changes and vulnerable to lethal injury. Cells are subjected to various osmotic changes during the cryopreservation process resulting in cellular damage. One of the major factors affecting the extent of cryoinjury is the rate of cooling; too rapid cooling will cause severe intracellular ice formation, but too slow can damage the cells due to high solute concentration. On thawing cells are exposed to lethal conditions of transition from hypertonic to isotonic solution causing extensive osmotic swelling, which can further deteriorate their viability. Inappropriate concentrations of cryoprotectants can cause the cell to shrink or swell beyond viable limits, thereby inducing osmotic shock and spermolysis [4].
5. *Hypothermic injury*
   Hypothermic injuries are different from freezing damage, osmotic stress, or CPA damage in which spermatozoa get injured by cooling from body temperature to 0 °C. This phenomenon is known as “cold shock” sensitivity and occurs when spermatozoa are cooled slowly, resulting in permanent loss of motility. Sperm plasma membrane is the primary site of cooling damage. Lipid phase transitions of plasma membrane may cause irreparable injury even after cells return to physiologic temperatures. Hypothermia affects membrane lipid composition, plasma membrane proteins, and cytoplasmic Ca^{2+} level. Aquaporin transmembrane water channel proteins (AQPs) may also be affected by hypothermia. Increase in the cytoplasmic Ca^{2+} level leads to capacitation-like changes, ionic leakage, and subsequent acrosomal exocytosis [23]. Intracellular concentration of sodium is regulated by sodium–potassium-dependent ATPase, which is highly sensitive to hypothermia. At low temperatures, reduction of its activity impairs proper functioning of a sodium pump, resulting in uptake of sodium ions from the extracellular milieu. Sodium ions were shown to be detrimental to the nucleus and DNA [24]. This mechanism may be responsible for hampering cell preservation at low temperatures.
6. *Impact on plasma membrane*
   Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease the fertility potential of the cells. Cryopreservation increases premature capacitation of spermatozoa [24]. These alterations may not affect motility but reduce life span, ability to interact with the female reproductive tract, and sperm fertility. Membrane fluidity has been positively correlated with the recovery of motile, viable spermatozoa from a cryopreserved sample. Membrane stability is affected by changes in temperature, osmotic stress, and volume changes due to movement of water and cryoprotectants. At molecular level, loss of membrane permeability is associated with modifications of lipid composition, specific lipid–protein interaction, and phospholipid asymmetry. Phase transitions of the membrane lipids impair the function of membrane proteins responsible for ion transport and metabolism. Glycerol is known to have a direct effect on the plasma membrane and to alter its fluidity by increasing the order of the fatty acid. Membrane swelling and acrosomal leakage associated with sperm cryopreservation has negative effects on sperm motility and velocity. The adaptability of the membrane to damaging effects of the freeze–thaw process

is markedly superior for spermatozoa with a high membrane fluidity [25].

7. *ROS (Reactive Oxygen Species)*
   Cryopreservation and thawing is associated with increased ROS production and decreased antioxidant level making spermatozoa more susceptible to ROS-induced damage. Cryopreservation alters mitochondrial membrane fluidity which leads to rise in mitochondrial membrane potential and the release of ROS [26]. Excessive production of ROS has been associated with loss of sperm motility, viability, membrane integrity, decrease capacity for sperm–oocyte fusion, and low fertility due to plasma membrane lipid peroxidation. The process of ROS production by both human spermatozoa and seminal leukocytes causes DNA fragmentation. Increased lipid peroxidation occurs due to higher production of ROS.
8. *Apoptosis*
   Apoptosis is programmed cell death, an underlying mechanism for normal spermatogenesis. When deregulated it causes male infertility. Said TM et al. demonstrated substantial increase in certain apoptotic markers after cryopreservation and thawing [27]. Cryopreservation induces apoptosis by activation of caspases and disruption of the mitochondrial membrane potential. Comparison of fresh versus cryopreserved spermatozoa has shown that cryopreservation significantly increases the percentage of spermatozoa with activated pan-caspases. Glycerol is claimed to activate caspases during cryopreservation.
9. *DNA Damage*

While the effects of cryopreservation on the fertilization capacity, motility, morphology, and viability of spermatozoa are well documented, the question of the possible alteration of sperm DNA integrity after freezing–thawing procedures still remains. There is no agreement in the literature on whether cryopreservation induces DNA damage or on the amount of damage. Significant alterations of sperm DNA integrity after cryopreservation–thawing have been reported in some studies [8, 28], whereas other studies have expressed a different opinion [29, 30]. These conflicting results can be explained firstly by the smaller sample size of the studies and also due to the use of (1) different freezing procedures, (2) different tests to evaluate the DNA integrity, and (3) different semen preparation techniques before cryopreservation (i.e., swim-up or density gradient centrifugation).

Compared with the conventional "slow"-freezing method, the newly developed technique of vitrification (cooling rate ~720,000 °K/min) seems to have more benefits with respect to sperm survival. Moreover, the entire freezing or thawing process only takes a few seconds.

Vitrification

"Vitrification" is a modern technique of sperm cryopreservation and is rapidly emerging as the future of cryopreservation. Although conventional slow-freezing method has played a vital role in the field of sperm cryopreservation, several reports have recently claimed that the vitrification of human spermatozoa gives higher recovery rates upon warming [31, 32]. Vitrification is the solidification of a solution at low temperature, not by ice crystallization, but by extreme elevation in its viscosity using high cooling rates of 15,000–30,0000 °C/min. The cooling of cells at this ultrahigh rate of freezing creates a glass-like state without intracellular ice formation. Thus, the term vitrification, which means "turned into glass" was first proposed by Luyet in 1937. In contrast to the conventional freezing, vitrification has a series of technological advantages useful for the practice: it renders the use of permeable cryoprotectants superfluous and, in addition, is much faster, simpler in application, and more cost-effective.

Principles of Vitrification

Vitrification involves exposure of the cell to high concentration of cryoprotectants for a brief period at room temperature followed by rapid cooling in liquid nitrogen. The cells are initially

pre-equilibrated in a cryoprotectant solution of lower strength (usually 10 %) resulting in dehydration of the cell and its permeation with the cryoprotectant. This is followed by a very short incubation (<30 s) in higher concentration of cryoprotectant solution (40 %) followed by rapid plunging into liquid nitrogen. The high osmolarity of the cryoprotectants results in complete dehydration of the cell. Since the cells are almost devoid of any water by the time they are immersed in liquid nitrogen, the remaining intracellular water, if any, does not form ice crystals. During warming, the entire process of vitrification of the cell is reversed. Cells are exposed in a stepwise manner to hypotonic solutions of decreasing strengths of sucrose to remove the cryoprotectant and gradually rehydrate.

Factors that Influence Successful Vitrification

The success of vitrification depends on the rate of cooling and concentration of cryoprotectant used. The rate of cooling in turn is influenced by the size and volume of the sample and vitrification solution. Concentration of the cryoprotectants is critical to the success of vitrification as high concentration of cryoprotectant can be toxic to cells.

The key elements that influence vitrification are:

- Cryoprotectants
- Carrier systems
- Techniques for better heat transfer

According to the literature, the critical cooling speed for the vitrification of pure water varies drastically, depending on the method used, from 10^7 to 10^{13} K/min. The presence of very high concentrations of CPAs substantially decreases the critical rate of freezing and warming; however, it is known to have a marked toxic effect [33–35]. It is possible to decrease cryoprotectant toxicity by the stepwise exposure of cells to precooled concentrated solutions [33, 36] and/or by reducing the amount of cryoprotectant and at the same time increasing cooling and warming rates [37]. This lowering of the CPA concentration consequently requires an increased rate of cooling and warming, which can be achieved by decreasing the volume of the cooled suspension and increasing the surface-to-volume ratio of the sample. The sample size can be minimized using different kinds of packages [37–39], microdrops [40], electron microscopic copper grids [41, 42], Cryoloops [39, 43], and nylon mesh [44].

To date, the results obtained with vitrification, in terms of survival of gametes, fertilization, progression of embryos to blastocysts, and pregnancy rates, are equal or even better than the conventional slow-freezing protocols. Its improved results may be attributed to avoiding potentially lethal intracellular ice formation. Similarly, the very fast warming rate serves to prevent the recrystallization that may otherwise occur in the supercooled vitrified glass state. Devitrification (especially intracellular growth of crystals) and mechanical damage (cracking) of the vitrified matrix during rewarming could be highly detrimental to the cell suspension; hence, warming should be undertaken as rapidly as possible. The cells might be more sensitive to the warming rate compared to the cooling rate during vitrification.

Vitrification of Human Spermatozoa with Cryoprotectants

Cryotop Method

Endo et al. 2011 [45]

Conventional freezing procedures and containers are not appropriate for spermatozoa from the testis because of their low number and poor in situ motility, and various types of container have been utilized to freeze small numbers of spermatozoa. Cryotop which is an open-type system consists of a fine polypropylene strip attached to a plastic handle and is equipped with a cover straw (Fig. 4.1).

Protocol

Individual sperm cells were transferred to a droplet of freezing medium (1 mL) on to the Cryotop strip using the intracytoplasmic sperm injection (ICSI) pipettes at room temperature. The Cryotop strip was then immediately placed at 4 cm (−120 °C) above the surface of the liquid nitrogen (LN_2) for 2 min and then directly exposed to sterilized LN_2. The Cryotop strip

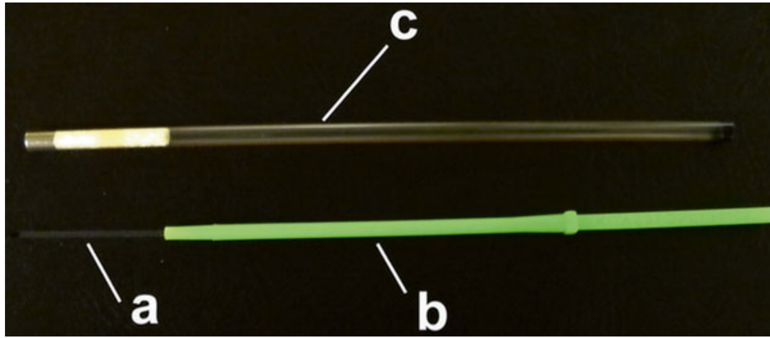

Fig. 4.1 Cryotop cryopreservation tool. A polypropylene strip (*a*) is attached to a hard plastic handle (*b*). After cryopreservation, the hard plastic cover (*c*) protects from not only physical damage but also virus contamination during storage in LN2 (Reprinted from Endo et al. [52], with permission from Elsevier)

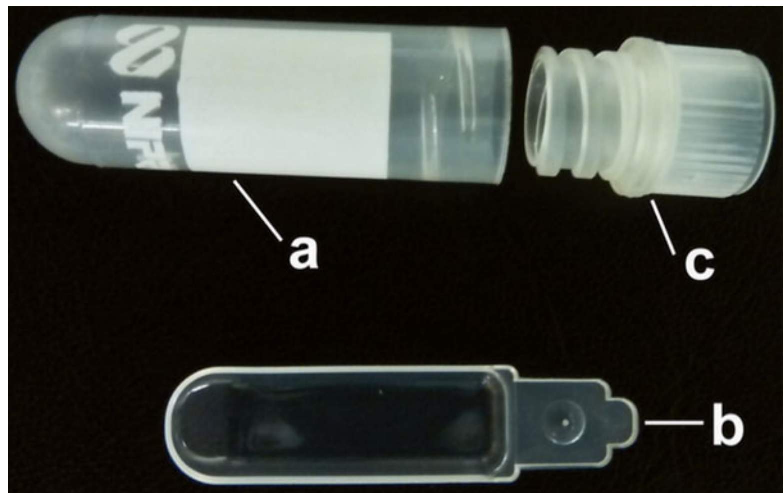

Fig. 4.2 The Cell Sleeper cryopreservation tool. The samples for cryopreservation were placed on a tray (*a*). After placing a tray into a cryotube (*b*), they were sealed with a screw cap (*c*) (Reprinted from Endo et al. [46], with permission from Elsevier)

was covered with the hard plastic straw during storage in the cryotank. For warming, the Cryotop strip was placed directly in a flat droplet of basic medium (2 mL), which was covered by oil in a Falcon 1006 dish (Becton Dickinson) at 37 °C. To prevent sperms from being left on the cryotop strip, it was further washed in two drops of 2 mL each. Each droplet was carefully checked, and recovered sperms were transferred to 8% polyvinylpyrrolidone (Irvine Scientific) drops. After being washed three times by ICSI pipettes, they were stored until ICSI procedures.

Outcomes

Freezing of motile single sperm obtained from ejaculates and the testes were evaluated for recovery rate (90.0 % vs. 95.0 %) and motility rate (44.4 % vs. 42.1 %), which were not significantly different. The survival rate was significantly higher when sperm were treated with sucrose rather than with glycerol (65.3 % vs. 37.3 %, $P<0.01$). These results demonstrated that Cryotop was a highly effective tool for the cryopreservation of a single spermatozoon, and sucrose was determined to be an efficient cryoprotectant.

Cell Sleeper Method

Endo et al. 2012 [46]

Cell Sleeper which is a closed-type system is a type of vial used as cell cryopreservation container, which is equipped with an inner tray and is sealed with a screw cap (Fig. 4.2).

Protocol

Ejaculates from infertile men were processed through density gradients and swim-up procedures. About 3 μl swim-up spermatozoa was carefully transferred to a droplet of 7 % polyvinylpyrrolidone in a Falcon 1006 dish. After 3–10 min of culture at 37 °C, individual motile spermatozoa were picked up using an ICSI pipette equipped with a micromanipulator and transferred to an oil-free droplet of 3.5 μl of SpermFreeze-based cryopreservation medium deposited on the tray. Immediately, the tray with the oil-free droplet was put immediately into a cryotube and cooled in LN_2 vapor for 2.5 min and then submerged in LN_2. The Cell Sleeper was mounted on a cane and stored in a cryotank. After warming the Cell Sleeper, the tray was taken out immediately from the cryotube and the droplet on the tray was covered with oil. The droplet was cultured at 37 °C for 2 min and observed in order to retrieve the spermatozoa.

Outcomes

When Vitrified in the count of five sperms per container, all sperms were recovered, and the viable sperm rate was 72 % when sperm were vitrified in 3.5 μl of the droplet. From these results, it was concluded that Cell Sleeper was a highly effective tool for the cryopreservation of small numbers of sperm and limited cells could be vitrified quickly and simply without significant loss.

Vitrification of Human Spermatozoa Without Permeable Cryoprotectants

Favorable results have been obtained in human spermatozoa after excluding permeable cryoprotectants from cryopreservation solutions, increasing the cooling rate and using carbohydrates, proteins, and other extracellular agents, to increase the viscosity of the surrounding medium of cells and prevent the formation of any intra- and extracellular crystals. It was shown that permeable cryoprotectant-free vitrification only with protein or in combination with sucrose as a non-permeable cryoprotectant provides a high recovery rate of motile cells and effectively protects the mitochondrial membrane and the DNA integrity of spermatozoa after warming. According to common point of view, the non-permeable cryoprotectants play the supporting role as permeable cryoprotectants. They bind to extracellular water and at the same time play antitoxic role decreasing the harmful properties of permeable cryoprotectants. In general, the inclusion of osmotically active, non-permeating compounds into the vitrification solution leads to additional rehydration of cells and, as a result, to decreasing toxic effects of the permeable cryoprotectants on intracellular structures. The non-permeable cryoprotectant sugars possess a unique property: stabilization of a cell membrane.

Survival of spermatozoa without permeable cryoprotectant could be due to:

1. Presence of large amounts of osmotically inactive water bound to several macromolecular structures such as DNA, histones, and hyaluronidase [47]
2. Presence of high-molecular-weight components in spermatozoa, which affects the viscosity and glass transition temperature of the intracellular cytosol [30]

Thus, vitrification of human spermatozoa in the absence of cryoprotectants is feasible.

Also, the application of this modified cryopreservation technique to human spermatozoa avoids the toxic effect caused by adding and removing of permeable cryoprotectants including the negative effects on the cells' genetic material.

Insemination Straw

Sanchez et al. 2012 [48]

A new technique for freezing sperm can dramatically increase the viability of frozen sperm. In addition, as the technique does not involve the freezing of seminal plasma, it holds out the possibility of allowing sperm from HIV+ men to be used without the danger of transmitting the virus.

Protocol

The sperm suspension obtained after swim-up is centrifugated. The sperm resuspended in 50–100 μl of vitrification solution containing 0.25 M sucrose and 1 % human tubal fluid–human serum albumin (HTF-HSA) is placed in 0.25 ml insemination straw (IE). The IE with spermatozoa was first placed inside a sterile 0.5 ml

insemination straw, which was hermetically closed by heat at both sides, and then plunged into liquid nitrogen. The warming is performed by quick direct submerging of 0.25 IE into 5 ml of HTF-HSA 1 % (pre-warmed to 37 °C).

Outcomes

Human spermatozoa cryopreserved without permeable cryoprotectants preserved relatively high motility rate (77.0 ± 2.5 %) with the ability to fertilize oocytes in vitro. This vitrification technique that preserves most of the sperm cell functions is an easy, speedy, and cheap alternative for sperm bank cryopreservation.

Vitrification of Human Spermatozoa Without Cryoprotectants

The conventional method of vitrification used to preserve large cells (embryos, oocytes), tissues, or organs requires high CPA concentrations with the consequent lethal osmotic and toxic effects. As a result, it has not been possible to successfully cryopreserve the osmotically sensitive mammalian spermatozoon by conventional vitrification. Advanced vitrification technology does not require the use of classic permeable cryoprotectants. This avoids the lethal effects of cryoprotectants toxicity and osmotic stress and the damage of plasmatic and mitochondrial membrane during equilibration with cryoprotectants and protects the plasmatic and mitochondrial membrane against lipid peroxidation and formation of reactive oxygen species and DNA damage.

Cryoloop Method

Isachenko et al. 2004 [32]

This is a new technique of ice and CPA-free cryopreservation (vitrification) by direct plunging of a sperm suspension into liquid nitrogen. After storage, warming is achieved by direct melting of the frozen suspension. By avoiding the use of the classic permeable cryoprotectants, the lethal effects of osmotic shock are prevented. The goal of the study was to compare the quality of spermatozoa in terms of motility, DNA integrity, and fertilization ability, cryopreserved using a very fast cooling rate (that leads to practically instant vitrification) by plunging into liquid nitrogen, with cryopreservation at a slower rate (but still fast in the conventional sense) by freezing in liquid nitrogen vapor before immersion into liquid nitrogen.

Protocol

Instant Vitrification and Warming

The prepared spermatozoa were loaded onto copper loops of 5-mm diameter by dipping the loops in a sperm suspension to obtain a thin film of 20 ± 2 μl. The loaded loops were then plunged into liquid nitrogen. After storage for a minimum of 24 h, the samples were thawed by plunging the loops into a 15-ml tube containing 10-ml standard preservation medium (SPM; Scandinavian IVF Science, Gothenburg, Sweden) at 37 °C under intense agitation. After warming five loops in one tube, the tube was placed in a CO_2 incubator for 5–10 min. Next, the spermatozoa were concentrated by centrifugation at 380 × g for 10 min, and the resultant pellet was resuspended in 100 μl of SPM and processed for further evaluation.

Freezing in Liquid Nitrogen Vapor

Spermatozoa were vitrified and thawed according to the procedure described above with the following modifications. Before plunging into liquid nitrogen, the loops were cooled for 3 min in liquid nitrogen vapor at −160 °C. This was achieved by placing the loop in a Styrofoam box (5 × 5 × 10 cm) containing a 0.5–0.8-cm depth of liquid nitrogen ~1 cm above the liquid nitrogen level.

Strategies to Improve Outcome of Sperm Cryopreservation

Cryosurvival rate of sperms is dependent on sperm preparation technique, cryoprotectant media, freezing and thawing protocols, and the quality of the native semen sample. In spite of new innovations in cryobiology, a gold standard method for cryopreserving human spermatozoa is yet to be determined, which will minimize the cryodamage and maximize the success rates in ART.

1. *Sperm selection technique*
   Incorporation of an appropriate sperm preparation technique may aid in improving post-thaw survival of spermatozoa. Swim-up and density gradient are widely used techniques for separation of functionally and morphologically competent sperms from the apoptotic one [49]. This preselection has been shown to improve sperm quality after thawing in terms of all the classic markers of quality including DNA integrity. Density gradient centrifugation is also superior than swim-up when it comes to isolating sperm with non-fragmented DNA [7]. But both density gradient centrifugation and swim-up techniques have been equally efficient in reducing the number of apoptotic sperms [50]. Higher number of quality sperms can be revived, if the apoptotic spermatozoa are removed prior to cryopreservation using these techniques.
2. *Increasing the surface-to-volume ratio of the sample*
   Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. The rate of heat exchange is governed by the temperature difference between the inside and the outside of the sample and by the extent of heat conduction. The latter depends strongly on the volume to surface ratio of the sample. Many techniques have been developed to reduce sample volume with an explosion of methods appearing in the literature during the last decade.
3. *Cooling and warming rates*
   The sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per min, depending on the container, the volume, the thermal conductivity, the solution composition, etc. In the surface methods, if the size of the drop (~0.1 μl) can be controlled, high cooling rate can be achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. Higher cooling rates can be achieved by quickly passing through the damaging vapor phase above liquid nitrogen.
4. *Viscosity of the media*
   Viscosity of the medium is defined by the concentration and behavior of various CPs and other additives during vitrification. The higher the concentration of CPs, the higher the glass transition temperature (Tg), thus lowering the chance of ice nucleation and crystallization. Different CPs and other additives have different toxicity, penetration rate, and Tg. The combination of different CPs is often used to increase viscosity, increase Tg, and reduce the level of toxicity.

Cryopreservation and Reproductive Outcome

Cryopreservation is widely known to raise impaired sperm motility and decrease fertilization rate through detrimental effects on membranes, acrosomal structure, and acrosin activity [50]. The freezing–thawing procedure of human spermatozoa may also be detrimental to the chromatin structure [18], leading to a potential risk of decondensation of the sperm nucleus after injection into the oocyte, thus reducing fertilization rate [51]. However, a cumulative effect of cryopreservation on sperm fertilization capacity is not definitely established. Considering the low fertilization rate and motility due to post-warming, fresh samples are preferred for conventional IVF and intrauterine insemination over cryopreserved sample. However, the considerations are different for ICSI because only a small number of motile sperms are required for successful fertilization.

Conclusion

Sperm cryopreservation is an ancient method of preserving male fertility in a way that will maintain sperm viability for a long period of time. Despite the adverse effects that cryopreservation exerts on sperms, it still remains one of the core methods of fertility preservation and much of its successful application seems to affect the reproductive outcome of ART. Vitrification is an emerging technology; it has both a great potential and a need for further developments. In contrast to conventional freezing, the vitrification

renders the need for special cooling programs in addition of permeable cryoprotectants. It is much faster, simpler, and more cost-efficient while still effectively protecting spermatozoa from cryoinjuries and does not require expensive equipment or special cooling procedures. Future studies are expected to concentrate on optimal freezing conditions to achieve better viability of cryopreserved sperms.

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5 Ovarian Tissue Vitrification for Fertility Preservation

Noriko Kagawa, Iwaho Kikuchi, and Masashige Kuwayama

Keywords
Ovarian tissue • Cryopreservation • Transplant • Oocyte • Vitrification • Cancer

Introduction

Ovarian Tissue Transplantation

The fairly recent development of ultrarapid vitrification has expanded the application of cryopreservation and dramatically changed the field of human-assisted reproduction [1]. However, cryopreservation of mature oocytes is not possible for prepubescent girls and is not suitable for cancer patients requiring immediate treatment because there is not enough time for adequate harvest of mature oocytes before such treatment. Cryopreservation of ovarian tissue has the potential to solve these problems and to restore patients' natural fertility after aggressive chemotherapy and/or radiotherapy.

In 2005, Silber and colleagues reported the first successful ovarian tissue transplantation between identical twins [2]. Dr. Silber then requested that we develop a new technique for ovarian tissue cryopreservation and autografting. We achieved efficient ovarian tissue cryopreservation by ultrarapid vitrification [3] and successfully applied the technique to a young, unmarried cancer patient. We also reported the first successful transplantation of vitrified ovarian tissue between human leukocyte antigen-matched sisters [4]. The recipient's cycles resumed 4 years after extensive chemotherapy and amenorrhea and 42 days after transplantation of the thawed ovarian tissue, which was achieved by minilaparotomy. To date, 41 live births (12 from fresh tissue transplantation and 29 from cryopreserved tissue transplantation) have been reported by 13 medical doctors worldwide [5]. Silber and colleagues have reported the greatest number of live births after ovarian tissue transplantation: 11 from fresh tissue transplantation and 3 from frozen tissue transplantation [5].

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A Strategy for Fertility Preservation in Cancer Patients

Ovarian Tissue Banking: Immature Oocyte Collection and Cryopreservation

The oocyte collection and cryopreservation procedure is carried out laparoscopically as follows: The operative field of view is secured under pneumoperitoneum, and peritoneal cavity features are confirmed. An 18-gauge Cathelin needle attached to a syringe is inserted transabdominally to reach the ovary and then into a small follicle on the ovarian surface. Follicular fluid is aspirated into the syringe, as for in vitro fertilization. The aspirated follicular fluid is mixed with culture medium, and immature oocytes (germinal vesicle-stage [GV] oocytes; Fig. 5.1) are collected from the resulting culture medium under microscopy and vitrified by Cryotec method (Fig. 5.2). Oophorectomy is routinely performed by the single-incision laparoscopic surgery (SILS™) method; that is, the ovary is freed from the peritoneal cavity by severing the ovarian arteries and veins and the proper ovarian ligament with the use of a LigaSure Blunt Tip laparoscopic sealer/divider (Covidien, Mansfield, MA). The ovary is carefully extracted in an Endo Catch™ specimen pouch (Covidien) through the incision in the umbilical region. The cortical membrane obtained from the harvested ovary is cryopreserved by the vitrification method, as described below. We collect GV-stage oocytes by puncturing and aspirating small follicles of the contralateral ovary at the time of surgery. We believe that this method is useful because it improves the potential for pregnancy after chemotherapy and because there is no possibility of reimplanting tumor cells, which can occur at the time of autotransplantation of cryopreserved ovary.

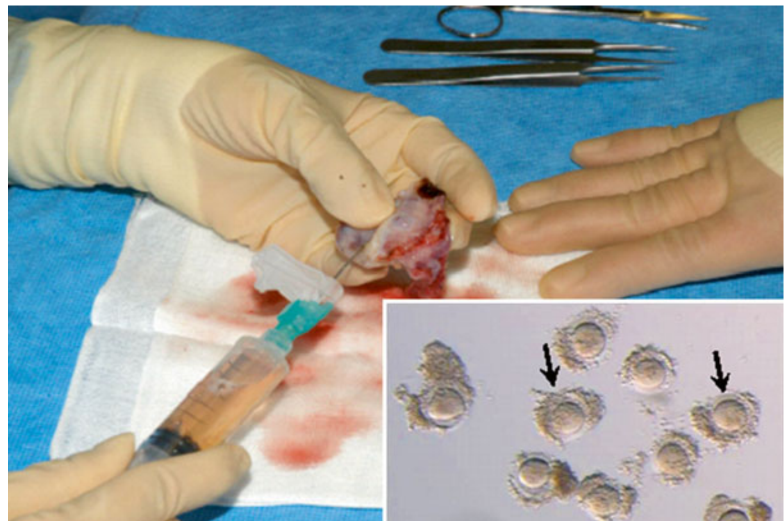

Fig. 5.1 Aspiration of immature oocytes from extracted ovary. Before cryopreservation, immature oocytes (see *inset* for magnified view) are obtained and partially denuded by pipetting with a glass needle before cryopreservation. In this case, 10–15 oocytes were collected from the patient, who was less than 35 years of age

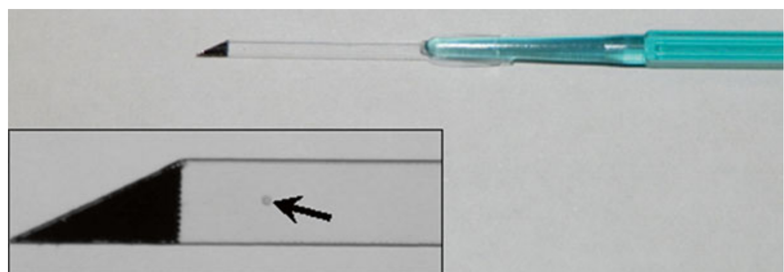

Fig. 5.2 Cryotec strip (vitrification device for human oocytes). Three to four human oocytes (*arrow*) are placed on the Cryotec strip for vitrification. These immature oocytes are partially denuded by glass pipetting before equilibration in vitrification solution

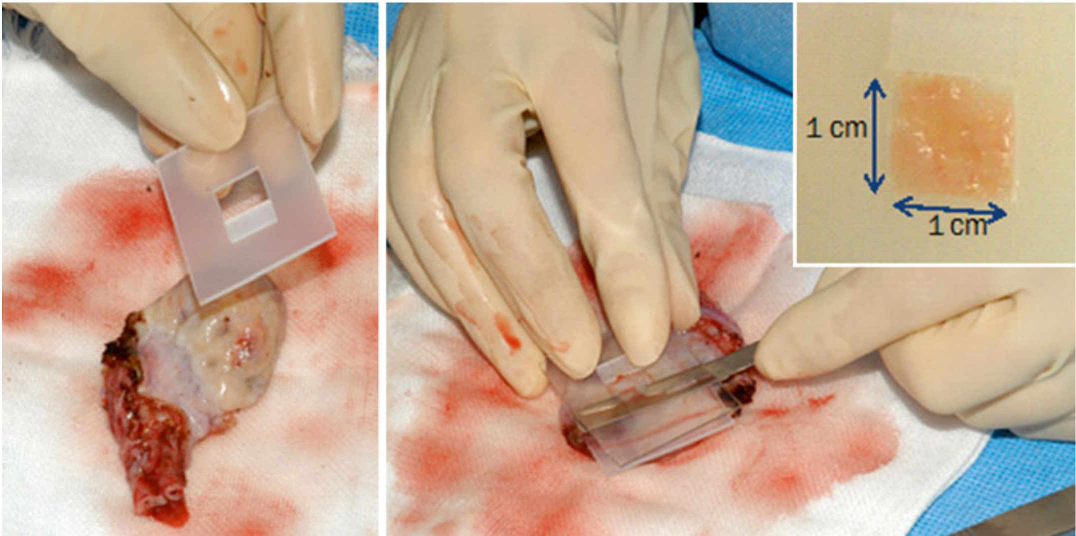

Fig. 5.3 Peeling of ovarian tissue cortex with a slicer. Ovarian cortical tissue containing primordial and primary oocytes is taken off in 1-cm × 1-cm strips with a special slicer that has square hole and allows for easy, quick, uniform slicing

The GV-stage oocytes are matured in vitro, fertilized in vitro, and transferred to the patient's uterus.

Vitrification of the Ovarian Tissue

Vitrification of the ovarian tissue is performed with a thin metal device and vitrification solution for ovarian tissue, as reported previously [3]. Briefly, ovarian cortical tissue containing primordial and primary oocytes is peeled away in 1-mm-thick slices (12 × 12 mm) (Fig. 5.3), equilibrated in 7.5 % ethylene glycol and 7.5 % dimethyl sulfoxide in handling medium (HEPES-buffered m-MEM supplemented with a high-molecular-weight compound) for 25 min, and then equilibrated again in 20 % ethylene glycol, 20 % dimethyl sulfoxide, and 0.5 M sucrose in handling medium (vitrification solution) for 15 min. Ovarian tissues are then placed with a minimum volume of vitrification solution onto a thin metal strip with small holes and submerged directly into sterilized liquid nitrogen (Fig. 5.4), after which the strip is inserted into a protective container and placed in a liquid nitrogen storage tank.

Clinical Results of Ovarian Tissue Cryopreservation by Our Team in Japan

Between 2007 and 2013, oocytes and ovarian tissues were banked for 45 patients with various types of cancer. An average of 10.6 oocytes, 6.3 oocytes, and 6.3 oocytes per patient were harvested before chemotherapy, after chemotherapy, and after GnRh-a treatment, respectively, from extracted and contralateral ovaries under IRB approval and informed consent. An average of 8.3 ovarian tissue sheets (1 cm × 1 × cm × 1 mm) were obtained from 1.2 ovaries per patient.

The Forefront of Ovarian Tissue Transplantation

Beginning in 2010, our clinical research collaborator, Juntendo University, which pioneered single-port laparoscopic gynecologic surgery as a SILS™ procedure that can reduce the size of the cicatrix, began to accept cancer patients desiring fertility preservation [6]. Patients who do not have much time before the start of chemotherapy can elect to undergo immature oocyte aspiration and

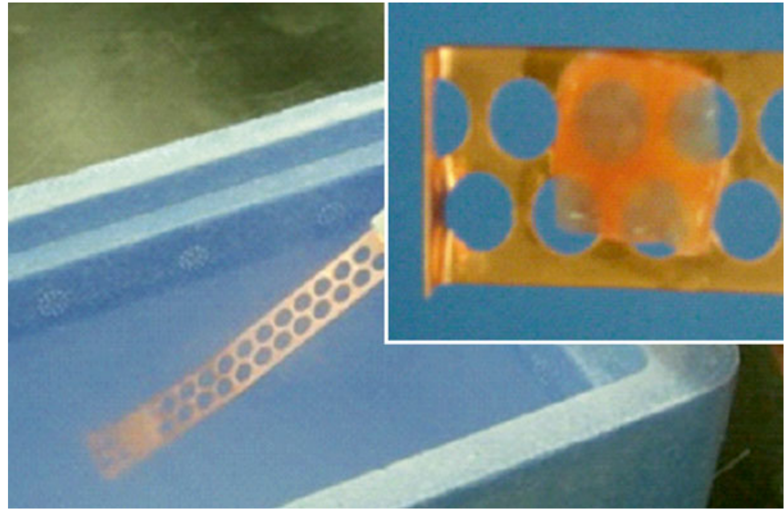

Fig. 5.4 Photograph of vitrified ovarian tissue submerged in liquid nitrogen (−196 °C) on the Cryotec strip. Ten to fifteen ovary pieces were put into the vitrification solution for equilibration, placed on a metal strip with small holes, and then cooled in liquid nitrogen The inset figure shows one ovarian tissue piece placed on the metal strip

laparoscopic oophorectomy. In addition, cancer survivors can request autotransplantation of their ovarian tissue by single-port laparoscopic surgery.

Minimally Invasive Surgery

Oophorectomy by SILS

For candidate patients scheduled to undergo chemotherapy, it is desirable that treatment of the primary disease be started as early as possible. We have begun to perform minimally invasive single-port surgery, which has attracted attention in recent years, and we have used this strategy for oophorectomy aimed at cryopreservation of the ovary [7]. Because reduced-port surgery, including SILS, requires fewer incisions than conventional laparoscopic surgery, postoperative recovery is faster [8, 9]. In cases in which the underlying disease is a hematologic disorder, SILS seems to be very useful because there is little perioperative bleeding. Another advantage is that the operation time is relatively short. However, with SILS, operative freedom is quite limited, and the surgical technique is somewhat difficult. Therefore, when intraoperative difficulty is anticipated, it is recommended that another port be added and that 2-port surgery be performed [10]. Given the presence of endometriosis, ovarian adhesions were anticipated in 2 of 23 cancer patients who, between 2009 and the present, underwent fertility preservation by means of ovarian tissue cryopreservation at Juntendo University Hospital, and such adhesions were indeed encountered. We therefore used 2-port surgery to ablate these adhesions. For single-port surgery, we use Covidien's SILS™ Port [11]. This port is appropriate for our purposes because it allows us to detach and extract the ovary via an approximate 2.5-cm incision in the umbilical region. As noted above, we use the Endo Catch specimen pouch (Covidien) to carefully extract the ovary.

Surgical Results

Operation time for each of the 23 patients was approximately 30 min, and estimated blood loss was less than 5 mL. All 23 patients were discharged from the hospital without postoperative issues and were able to undergo treatment, such as chemotherapy, for their primary disease according to predetermined schedules and without difficulty. The mean numbers of ovarian cortical tissues and GV oocytes collected were 10.1 and 16.3, respectively. Fewer GV oocytes were collected from patients who had endometriosis than from those who did not. Currently, all 23 patients are alive and receiving treatment for their primary disease.

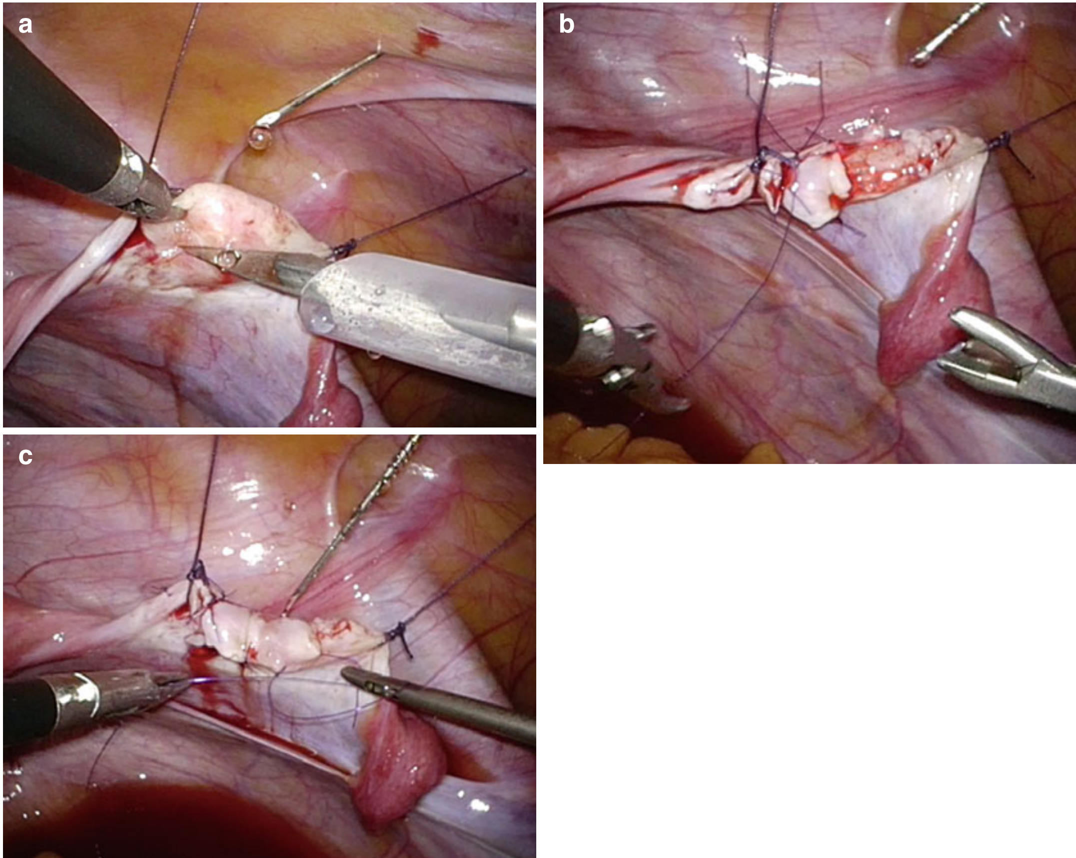

Fig. 5.5 Vitrified ovarian tissue transplantation by single-port surgery. One piece of ovarian tissue was examined pathologically to confirm the absence of tumor cell invasion. The capsule of the remaining right ovary was incised with a laparoscopic cold knife and scissors while heparin saline was instilled to avoid stoppage of blood flow. A base 2 cm × 1 cm in size was created on the remaining ovary (**a**). Two thawed ovarian pieces were laparoscopically sewn and fixed (**b**, **c**) to the base with 5-0 absorbable sutures

Ovarian Tissue Transplantation by SILS in a 26-Year-Old Patient Who Underwent Aggressive Chemotherapy and Bone Marrow Transplantation for Malignant Lymphoma

Before ovarian tissue transplantation in a patient who had been treated for malignant lymphoma, a piece of vitrified ovarian cortex was thawed and examined pathologically to confirm the absence of tumor cell invasion. The capsule of the remaining ovary was incised with a laparoscopic cold knife and scissors while heparin saline is instilled to prevent stoppage of blood flow, and a base, 2 × 1 cm in size, was created (Fig. 5.5a). Two thawed ovarian pieces were laparoscopically sewn and fixed to the base with 5-0 absorbable suture material (Fig. 5.5b, c). The assistant controlled the scope, placing its tip between the needle holder and forceps, and a stable view was maintained. The operation time was 2 h 21 min, and estimated blood loss was 50 mL, allowing hospital discharge without problems on the day after surgery. After about 4 months (on postoperative day 138), transvaginal ultrasonography detected a 7-mm ovarian follicle. In addition, the patient's estradiol level had risen to 94 pg/mL. On postoperative day 173, the ovarian follicle had increased to 10 mm in diameter (Fig. 5.6), and the patient's estradiol level had risen to 101 pg/mL. The ovarian follicle growth and rise in estradiol suggested recovery of ovarian function. However, because the patient does not yet wish to have children, she has requested ongoing follow-up.

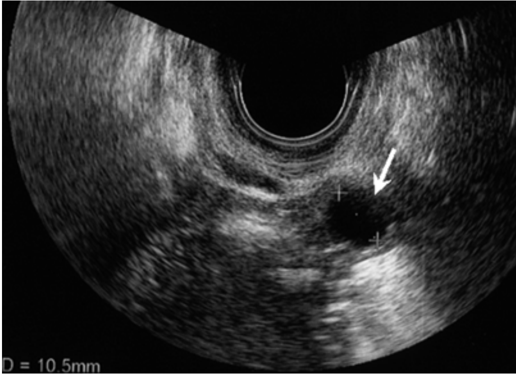

Fig. 5.6 Transvaginal ultrasonogram of follicle development after vitrified ovarian tissue transplantation by SILS. On postoperative day 173, the ovarian follicle had increased to 10 mm in diameter (*arrow*). The patient's estradiol level had risen to 101 pg/mL. The follicle growth and rise in estradiol suggested recovery of ovarian function

Testimonials of Cancer Patients Who Have Undergone Fertility Preservation by Means of Ovarian Tissue Cryopreservation

A 31-year-old lymphoma survivor from the USA told us, "You know, I feel so fortunate that I had cancer. My girlfriends in their 30s are all worried about their biological clock, but I have the ovary and oocytes of a 19-year-old and am, ironically, more fertile than they are."

A 26-year-old lymphoma patient in Japan responded to a written survey as follows:

Question 1: How did you feel, and what did you think is/are the advantage(s) of cryopreservation?

- Before consulting the hospital:
  I felt anxiety and shock at the possibility of infertility.
- After consulting the hospital:
  I was relieved that pregnancy might be possible.
- After surgery:
  I could freely concentrate on treatment and was surprised at how small the scar was and how little pain I felt.

Question 2: Did you have any concern or complaint/dissatisfaction regarding cryopreservation?

- Before consulting the hospital:
  I was concerned about having a scar on my stomach and about pain after surgery.
- After consulting the hospital:
  I was worried whether my eggs/ovary could be taken out properly.
- After the surgery:

I had no concerns or complaints.

Question 3: Do you have any other comments?

If this treatment can be covered by the National Insurance, more people will probably consider it. If a patient does not have enough money, she will have to give up this option. To make more women happy, the National Insurance should cover this treatment.

Discussion

The case of the 26-year-old woman described above is believed to be the very first case of ovarian cryopreservation by vitrification and ovarian capsule back-transplantation for a patient requiring treatment for a malignant tumor as well as the first reported use of reduced-port surgery as a grafting technique. The procedure was based fundamentally on the previously reported mini-laparotomy approach to ovarian tissue transplantation [2]. Cryopreservation by vitrification is more appropriate than slow freezing for frozen storage of relatively large tissue samples, and the cell survival rate is high. Transplantation of large tissue pieces generally requires suture manipulation. In the case described, however, an operative procedure approximating microscopically controlled surgery was successfully performed with a high-definition (HD) scope. The HD scope is required for precise suturing. In addition, as in microscopic suturing, a very thin thread, 5-0, is required. Silber placed a microscope just outside the open surgical area and succeeded in the first attempt at ovarian vitrification and subsequent transplantation [5]. Kikuchi et al. used the HD system under reduced-port laparoscopic surgery and achieved ovarian cryopreservation by vitrification and ovarian capsule back-transplantation for patients requiring treatment for a malignant lymphoma [12].

We hope that this operative procedure will aid women concerned with loss of ovarian function due to chemotherapy and help improve their

quality of life. The goal of the procedure is to furnish these women with strength and happiness. With good cooperation between oncologists and specialists in gynecologic laparoscopic surgery, ovarian tissue banking can be achieved safely. Although chemotherapy may significantly reduce the number of normal oocytes, research into new techniques, such as activation and development of oocytes in tissue cultures, is in progress. New techniques may provide even safer ways of producing oocytes other than by ovarian tissue transplant.

It is hoped that our research will help restore the full health of women who have undergone cancer treatment as well as the ability of these women to achieve pregnancy after treatment.

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Clinical Applications of Vitrification

6

Pankaj Talwar and Ved Prakash

Keywords
Cryopreservation • Vitrification • Tissue-engineered constructs, cryoprotectant agents • Stem cell

Introduction

Slow freezing and vitrification are two presently available methods in the reproductive laboratories for cryobioreposition [1]. Slow freezing is a time-honored technology pioneered in the early 1970s, which preserves the biological samples using controlled freezing rates, thus avoiding the intracellular ice formation and also minimizing structural damage to the cell membrane [2], cytosolic contents, and cytoskeleton [3].

The vitrification phenomenon first generated interest way back in the 1890s [4], but the clinical application of the technology for biopreservation of cells in suspended animation state was first acknowledged by Luyet and Gehenio in 1937 [5] (Table 6.1).

Here as the glass transition temperature of the solution is reached during the cooling process, the elevated viscosity of the amorphous glass conserves the natural disorder of molecules existing in liquid. This new state halts all chemical reactions and molecular motions, leading to immediate metabolic inactivity [6]. Thus, the

Table 6.1 Comparison of vitrification with slow-cooling procedures

	Vitrification	Slow cooling
Duration out of incubator	10 min	3 h
Prolonged temperature shock	None	Yes
Rate of cooling	15,000–30,000 °C/min	1 °C/min
Cryoprotectant concentrations	(6–8 M)	(1.5 M)
Cryodevices	Vitrification devices	Straw
Volume of cryoprotectants	1–2 μl	Larger volume
Equipment and running costs	Inexpensive	Expensive

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cells and tissues endure the extreme cooling process without any substantial damage subsequent from ice-crystal formation.

Table 6.2 Methods and objectives of vitrification/slow freeze protocols

	Objectives of the procedure	Steps of the procedure
1	To arrest the metabolism of the cells reversibly	Exposure to the cryoprotectant and removal of intracellular water to achieve dehydration
2	Structural and genetic integrity to be maintained	Slow/rapid cooling of the cells to subzero temperatures (−196 °C)
3	Accomplishing satisfactory survival rates after warming	Storage of the specimen at −196 °C
4	Maintain developmental competence post thaw	Thawing/warming by gradual rehydration
5	Reliable and reproducible technique	Dilution and removal of the cryoprotectant
6	Applicable to a large number of tissues with minimal variation	Recovery to a physiological environment

Later whenever required the vitrified/frozen cells and tissues are warmed/thawed with resumption of the biological activity (Table 6.2).

Throughout the whole cryopreservation process, cryoprotectant agents [CPAs] are necessary to protect cells from cryoinjury, which occurs while decreasing the temperature of the specimen due to intracellular ice formation. Cryoprotectants also act as osmotic buffers [7] and avert harmful critical electrolyte concentration gradients [7] harming the cells. These also act as membrane stabilizers thus stabilizing the integrity of the cells [8].

Common examples of extensively used CPAs include dimethyl sulfoxide (DMSO), 1,2-propanediol (PROH), ethylene glycol (EG) [9], and sugars like sucrose, trehalose, and mannitol (Table 6.3).

It is recommended to use the minimum sample volume (<1 μl) during vitrification to reduce the adverse effects of high CPA concentrations and to increase the rate of cooling, thus preventing cellular damage. Nano- and microscale technologies have now demonstrated that Vitrification using nano-volumes promises to transform biorepository technologies when to applied to modern medicine [10, 11].

Table 6.3 Types of cryoprotectants and their mode of action

Types of cryoprotectants	Mode of action	Examples	Biophysiological effects
Permeating	Compounds that readily permeate the plasma membranes of cells	Dimethyl sulfoxide (DMSO), propylene glycol, glycerol	These molecules form hydrogen bonds with water molecules and prevent ice crystallization At low concentrations they lower the freezing temperature of the resulting mixture However, at high enough concentrations, they inhibit the formation of the characteristic ice crystal and lead to the development of a glass-like solid, the so-called vitrified state in which water is solidified, but not expanded
Non-permeating	Large molecules that remains extracellular	*Sugars* (sucrose and trehalose)	When used in combination with a permeating cryoprotectant, it can reduce the required permeable *CPA* (*cryoprotective agents*) concentration and enhance the glass transition process
		Polymers (polyvinylpyrrolidone and polyvinyl alcohol)	This further assists the permeating cryoprotectant in preventing ice-crystal formation

Vitrification in Nature

Though the majority of living organisms are composed of large amounts of water which may sustain freezing injuries, few of them have adaptive capabilities and are able to withstand freezing. There is wide variation in the amount of freezing they can tolerate during cooling [12] which depends upon their endogenous antifreeze mechanism.

Arctic frogs and insects naturally generate glycerol or glucose in their livers which work as cryoprotectants. Such substances can reduce ice formation and lower the freezing point of the intracellular compartment acting as antifreeze molecules. The glucose is released in large quantities at extremely low temperature when a distinctive form of insulin is secreted which directs this additional glucose or glycerol to enter the cells. When the arctic frogs and insects rewarm during warm conditions, the extra glucose leaves the cell and is eliminated. Similarly the arctic fish utilizes antifreeze proteins (AFP), as cryoprotectants. Also the arctic beetles (*Pterostichus brevicornis*) routinely sustain temperatures below −35 °C, thus surviving the harsh winters.

Vitrification Versus Slow Freezing

Cryoprotectants are used at relatively low concentrations (1.5 M) [13] in slow freezing technique, which is a standard method for cell and tissue freezing [14]. Unfortunately cells undergoing slow freezing processes sustain cryoinjury, due to the configuration of ice crystals, hyperosmolar environment, and dehydration [15] (Tables 6.4 and 6.5). Vitrification is the transformation of the liquid phase directly into a glass-like solid. It offers improved clinical outcomes [16] by the avoidance of ice crystals and subsequent increase in ionic strength of unfrozen concentrated solutions [17, 18].

Vitrification techniques require higher CPA concentrations (6–8 M) and cooling rates (≈ −1,500 °C/min) compared with slow freezing methods. Rapid cooling can be achieved by immersing cells/tissues directly into liquid nitrogen (−196 °C).

High CPA concentrations used in vitrification technology lower the freezing point of the solution and increase the viscosity of the vitrification media. Nonetheless, it is known that these high CPA levels can cause osmotic shock and toxicity to cells resulting in alterations of the cytoskeleton [19] and spindle and chromosome scattering [20].

Carrier-Based Vitrification Systems

We have just understood that an increase in the cooling rate of the cells/tissues facilitates cells to pass through the phase transition temperature swiftly, thereby diminishing cryoinjury to the cell membrane and cytoskeleton [21].

Increasing the cooling and subsequent warming rates also leads to vitrification at lower cryoprotectant concentrations, thus lessening the unfavorable toxic and osmotic effects to the cells and tissues [22]. To tackle these challenges, various vitrification carrier systems (open and closed) have been designed (Table 6.6).

Open Vitrification Devices

Conventional Plastic Straw

This is an open vitrification carrier used for cryopreservation of embryos and oocytes [23] and provides a cooling rate of 2,500 °C/min and a warming rate of 1,300 °C/min. Being a plastic straw, however, this open carrier device fills in a large sample volume (~45 μl) [24], and with a thick wall it is hard to achieve a rapid cooling rate, and thus high CPA concentrations are required for the vitrification. To reduce the vitrification sample volume, several other methods have been developed such as open pulled straw (OPS) (~1 μl) [25] and hemi-straw systems (~0.3 μl), which have helped in reducing the chances of cryodamage as the cooling and warming rates (OPS 16,700 and 13,900 °C/min; hemi-straw >20,000 °C/min) were achieved as compared with the conventional plastic straws.

Table 6.4 Cryodamage and cell freezing

Structure	Types of damage
Nucleus and nuclear envelope (*NE*) *Role*: *DNA* (*deoxyribose nucleic acid*) replication, transcription, *RNA* (*ribonucleic acid*) processing, and ribosomal subunit assembly	The *NE* is made up of the outer and inner membranes having nuclear pore complexes and lamina Cryopreservation can affect the structural integrity of the NE and thus affect DNA replication and transcription
	Microtubules
Cytoplasm	Microtubules comprise of polymerized tubulins and are essential components of the cytoskeleton of the oocytes. These form the spindle apparatus in oocytes, which is responsible for spatial organization and subsequent migration of chromosomes during meiotic divisions. Cryopreservation of oocytes poses grave damage to the microtubules after thawing. Fertilization of oocytes with disorganized spindles could lead to non-fertilization, chromosomal aneuploidies, and cleavage arrest
Role: Protein synthesis, cytoskeleton formation and support	*Microfilaments* Microfilaments are components of the cell cytoskeleton and are composed of polymerized actin. In human oocytes microfilaments have been found organized in a uniform layer enveloping the cortex. During oocyte maturation, microfilaments play a vital role in polar body extrusion, pronuclear body migration, intracellular movement of organelles, and cell division Cryopreservation may affect microfilament function leading to problems in above functions Mitochondria Mitochondria play a vital role in the metabolism of the essential oocyte cytoplasmic organelles by providing adenosine triphosphate. These play an important role in fertilization and preimplantation embryo development. Mitochondrial swelling and their abnormal distribution have been observed after oocyte cryopreservation by conventional freezing, and vitrification
Zona pellucida (*ZP*) *Role*:	The zona pellucida is a glycoprotein membrane surrounding the oolemma of oocytes and early embryos. It is composed of three glycoproteins *ZP1*, *ZP2*, *and ZP3*
Plays a critical role in the fertilization process and blockage of polyspermy following initial penetration by the spermatozoon	Cortical granules are diffusely localized throughout the cytoplasm, and migrate to the oocyte cortex after fertilization of the oocyte. Their exposure to cryoprotectants, DMSO (dimethyl sulfoxide) and 1,2-propanediol, during the freeze-thaw cycle in oocyte cryopreservation leads to premature cortical granule release and zona hardening thus inhibiting sperm penetration and fertilization

Glass Capillaries

When used as a vitrification carrier device, it offers controllable cooling and warming rates using different diameters. It has been documented that capillaries with varying diameters of 440 μm–2 mm diameter have cooling rates of 12,000–2,000 °C/min and warming rates of 62,000–5,000 °C/min, respectively. It is clearly noted that larger diameters have lower cooling and warming rates. The glass capillaries have been used for cryopreservation of bovine oocytes [26] and embryos [27]. This method resulted in comparable hatching rates compared with those using the OPS technique (glass capillary 19 %; OPS 27 %; control 80 %), suggesting no critical difference in the cooling/warming rates relative to OPS. This method is not commonly used in clinical practice.

Table 6.5 The type of cryo-cellular injuries

Type of injury	Outcome
Chilling injury (−5 to −50 °C)	Causes irreversible changes in cellular structures – lipid droplets, protein, lipid-rich membranes, and micro-tubuli of the meiotic spindle
	Oocytes from different mammalian species and developmental stages contain different amounts of lipid and may have different sensitivities to chilling injury
	Chilling injury to the oolemma in the *MII* (*metaphase 2*) stage oocyte is more serious than in the early stages of embryos
Osmotic shock during equilibration (−5 to −50 °C)	Both permeable and non-permeable cryoprotectants may induce toxic and osmotic injury to the cell. Toxicity is usually proportional to the concentration of the cryoprotectants, temperature of equilibration, and the time of contact of oocyte and the *CPA* (*cryoprotective agent*)
	Grave deformation of the oocytes' shape occurs when they are exposed to the cryoprotectant solutions as a result of the osmotic effect. Fortunately in spite of the somewhat frightening morphological sight, human oocytes seem to tolerate these deformations and recover well during vitrification
Hardening of the zona pellucida	Premature release of the cortical granules due to cryotoxicity and low temperature may occur leading to zona hardening**.** ICSI (intracytoplasmic sperm injection) can circumvent this condition
Fracture injuries (−50 to −150 °C)	Zona fracture is a known consequence of all cryopreservation techniques and occurs frequently in both oocytes and embryos
	Fortunately embryos may survive some level of cell damage, while for the oocyte, any injury at this level is nearly nonreversible
	In a closed vitrification/freezing system, the extreme pressure changes caused by rapidly cooling or warming air bubbles induce dislocations in the partially solidified solution and injure the zona pellucida
	In the open vitrification systems, such mechanical forces are almost completely avoided. The extremely small volume of solutions used also minimizes the chance of fractures
Safe zone (−196 °C)	Very safe temperature for storage of the gametes since the cells here are in a suspended animation. Terrestrial background radiation levels of 0.1 *c Gy* (*centigray*)/year is too low a radiation dose to harm the cryopreserved oocytes

Quartz Microcapillaries

The thermal conductivity of the material of the carrier can alter the cooling rates of the device and the loaded specimen. Quartz carriers have been recommended for vitrification [28, 29] given their higher heat transfer rates (cooling rate of 250,000 °C/min) can be achieved compared to conventional glass and plastic cryodevices. The use of thin-walled quartz microcapillary devices with an outer diameter of 0.2 mm and wall thickness of 0.01 mm has led to ultrafast cooling rates of up to 250,000 °C/mm [30]. Higher cooling rates lower the cryoprotectant concentration and further reduce cell toxicity. Various studies have revealed better survival of the cells with this technique as compared to conventional straws, OPS, and electron microscopy grids.

Cryoloop

It is one of the commonly used devices to vitrify embryos and oocytes, and it has repeatedly demonstrated increased cell survival as compared to capillary-based devices [31]. The carrier here consists of a small nylon loop (0.7–1.0 mm in diameter) mounted on a stainless steel holder inserted into the lid of a cryovial. The sample is placed on the Cryoloop, which has a thin layer of CPA film, and then the loop with the sample is directly immersed in the liquid nitrogen. The loop is then fixed in the cryovial base, which is already filled with liquid nitrogen. Since the sample volume is limited to the loop size (<1 μl), cooling rates as high as 700,000 °C/min can be achieved [32, 33].

Cryotop

It is an open vitrification carrier, which has a polypropylene strip, attached to a holder accompanied with a protective sleeve. Here the sample volume (<0.1 μl) is loaded on top of the filmstrip. Subsequently the excess solution is aspirated, leaving behind a thin layer

Table 6.6 Cryodevices and their functionality

Cryodevices	Salient features
Standard 0.25 ml insemination straw	Relatively large amount of solution required to form a stable column in the straw and thick plastic wall of the sealed straw presents a considerable thermoinsulating layer
Volume of loaded solution: >5 μL (microliter)	Cooling rate I ≈2,500°/min Warming rates ≈1,300 °C/min
Open pulled straw (OPS) technique	Loaded with a tiny amount of solution containing the sample and plunging it into the liquid nitrogen
Glass micropipettes (GMP) Superfinely pulled OPS (SOPS) Gel-loading tips Volume of loaded solution: <1 μL	The achievable cooling and warming rates with these tools may be as high as 20,000 °C/min
Cryoloop-A 20 μm nylon loop, 0.5 mm in diameter, mounted on a 20 mm steel tube attached to the lid of the cryovial	Thin solution film bridging the hole of the loop is formed, and the oocytes and embryos are loaded onto this film
Volume of loaded solution: ≈1 μL	The film remains intact during immersion into liquid nitrogen, the solution volume is negligible, accordingly the cooling and warming rate may reach the estimated level of ≈7,00,000 °C/min, and the storage may be done in cryovials
Minimum drop size (MDS)	Very small droplet containing the sample is placed onto a solid surface and immersed into the liquid nitrogen
Volume of loaded solution: <0.5 or even 0.1 μL	Place small drops on precooling metal surface instead of liquid nitrogen for cooling. Originally, a metal block immersed into liquid nitrogen was used, but eventually a commercially available technique has also been marketed by CMV (*CryoLogic Vitrification Method*), CryoLogic, Australia
Cooling VIT Master (Vitrification Master) using liquid nitrogen slush	Aims to eliminate the vapor coat that arise around the sample in the liquid nitrogen for cooling
High security vitrification kit (HSV kit) Volume of loaded solution: <0.5 μl	This high security vitrification kit (HSV kit) makes it possible to place a microdroplet of cryoprotectant containing the embryos in the gutter of a capillary before inserting it in a mini-straw. It is heat sealed using a special welder, which ensures a leakproof seal
Cryotop	To protect the filmstrip and the sample cryopreserved on it, a 30 mm long transparent plastic cap is also provided to cover this part during storage in liquid nitrogen. The device is sterilized and should be handled under aseptic conditions and only for one cycle of vitrification
The Cryotop consists of a 0.4 mm wide, 20 mm long, 0.1 mm thick flexible filmstrip attached to a rigid plastic handle	Cooling rates ≈23,000 °C/min
Volume of loaded solution: 0.1 μl	Warming rate ≈42,000 °C/min
Cryotip A plastic straw container that can be sealed as a closed device to hold gametes or embryos during cryopreservation Volume of loaded solution: 1 μl	Cooling rates ≈1,200 °C/min Warming rate ≈2,400 °C/min
Electron microscopy grids (EMG)	Vitrification is done on small (several millimeters) copper discs called *grids* cast with a fine mesh. The mesh dimensions can vary a lot depending on the intended application, but is usually about 15 squares per millimeter (400 squares per inch)
Volume of loaded solution: <1 μl	On top of this grid, a thin layer of carbon is deposited by evaporating carbon graphite onto it. Thin carbon film on the grid holds the sample during the procedure Oocytes are placed on the electron microscopy grid and directly plunged into liquid nitrogen (LN_2) Cooling rates ≈150,000 °C/min Warming rate ≈150,000 °C/min

sufficient to cover the cells to be cryopreserved. The minimum sample volume used for this approach enhances the cooling rate up to 23,000 °C/min and warming rate up to 42,100 °C/min [32].

The Cryotop method has demonstrated higher efficiency for oocyte vitrification as compared to plastic straws and OPS with improved fertilization and blastocyst development with minimal cryodamage [34].

Electron Microscopy Grid

EMG has been used to vitrify samples with a volume less than 1 μl, with cooling and warming rates of approximately 150,000 °C/min [35, 36]. This open device has been used for vitrification of bovine blastocysts [37] and has demonstrated an increased hatching rate (68 %) compared with plastic straw vitrification device (53 %).

Cryoleaf

This is an open system vitrification carrier having a polypropylene plate for loading the embryos and a green sleeve, which can lock over the plate after vitrification process. This assembly is further covered by plastic sheath for the protection of the embryos.

Cryotech

An open style method is recognized as easy and simple for all. Cryotech is a special device that allows to minimize the volume that will be cooled and warmed with a very good result of survival for oocytes and all the stages of preimplantation embryos. It is a compact open system container by monobloc casting. It has a longer and wider handle and sheet for easy handling, writing, and loading the oocytes and embryos. It comes with a plastic cap to protect oocytes and embryos from not only physical damages but also contaminations of any kind during the storage in liquid nitrogen. One should place the oocyte or embryo near the end of the sheet with a minimal volume of VS2 (1 oocyte or embryo per droplet is recommended). After that immediately submerge the Cryotech into fresh liquid nitrogen. Put the straw cap on the Cryotech in the liquid nitrogen.

Surface Vitrification Devices

CVM Fibreplugs

These are designed to vitrify samples in minimal volumes and avoid contact between the specimen and liquid nitrogen. There is no thermal barrier around the surrounding specimens as they are not frozen in straws/open cryodevices but in a microdrop at one end of a fine fiber, which has a very low thermal mass and high thermal conductivity. Vitrification is performed on the prechilled solid surface of a specially designed block, which ensures ultrarapid cooling of specimen.

Closed Vitrification Devices

Cryotip is a closed vitrification device that has a narrow capillary, which can be heat sealed at both ends after cell loading to provide a closed system. The cryodevice offers lower cooling and warming rates (12,000 and 24,000 °C/min, respectively). As compared with the Cryotop method, survival of human blastocysts, pregnancy rates following vitrification did not demonstrate a significant difference [38].

High Security Vitrification (HSV) Straw

The HSV (high security vitrification) kit is composed of three parts: a high security ionomeric resin straw, a capillary tube with a preformed gutter attached to a colored handling rod, and a blue plastic insertion device. A sample is loaded in the gutter in minimal volume, and it is inserted in the outer straw, which is later sealed. This technique gives complete protection from cross-contamination.

Closed Pulled Straws

They have been designed to avoid potential microbial cross-contamination from direct contact with liquid nitrogen. Improved survival (79 %) and spindle morphology conservation have been shown with this method compared with OPS for the vitrification of mouse oocytes [39].

Limitations of Carrier-Based Vitrification Approaches

Carrier-based vitrification systems both open and closed have improved the survival of cells undergoing cryopreservation compared with conventional plastic straw devices. The technology involves skilled embryologists, manual handling, and thus compromised outcomes in certain situations. Nowadays, nanoscale droplet vitrification is being contemplated, and it offers a carrier-free approach, which involves generation of cell encapsulating cryoprotectant drops followed by their direct injection into liquid nitrogen [40]. No vitrification carrier device is needed in this technique, and it offers higher cooling and warming rates through direct contact with liquid nitrogen.

Carrier-Free, Droplet-Based Vitrification Approaches

Droplet-Based Vitrification

Droplet-based vitrification encompasses deposition of the cryoprotectant cell suspension in the form of microdroplets into the liquid nitrogen [41, 42]. The ejection technology can generate droplets of varying sizes at a rate of nearly 1,000 droplets per second [43, 44]. The acoustic ejection system, a variant of automated ejectors, can generate small droplets of volumes of <1 nl [45, 46]. Smaller droplets permit the vitrification to occur at higher cooling and warming rates using significantly reduced CPA concentrations (~1.5 M), thus minimizing the osmotic and cryobiological stress to cells. Various somatic cell types – hepatocytes, fibroblasts, and cardiomyocytes – have been vitrified using this technology with high survivability (>89 %) after warming [40, 41].

However this technology has its inherent disadvantages. Dropping a CPA suspension directly into liquid nitrogen leads to drifting of the droplet on the surface of liquid nitrogen surrounded by liquid nitrogen vapor blanket, which is known as the Leidenfrost phenomenon [41, 47]. The vapor blanket hypothetically becomes the gridlock for effective cooling of the specimen. Despite this drawback, carrier-free vitrification technology can still provide higher heat transfer rates than carrier-based systems, if smaller size droplets are used.

Solid Surface Vitrification (SSV)

This method vitrifies specimen without direct contact with liquid nitrogen. Here cells suspended in a small droplet of cryoprotectant solution are dropped onto a precooled metal surface (alloy block) cooled by liquid nitrogen or liquid nitrogen vapor followed by their rapid immersion into liquid nitrogen. The main advantage of this technique is the potential to achieve higher cooling and warming rates due to the absence of a bulky carrier and vapor effect. The technology has been applied to freeze oocytes generated in bovine and porcine practice [47–49].

Applications of Vitrification in Cryopreservation of Cells and Tissues

The concept of successful freezing depends upon the type of the cell, its water content, and its surface area. The type of cells in the sample also matters as these cells may have different permeability coefficient. The cells, which have large water content and small surface area, always pose a challenge for the cryobiologist. Majority of the human cells can be cryopreserved using slow freezing methods with nearly 80–90 % recovery of the function but have limitations when freezing cells with low surface area to volume ratio.

Newer Applications of Vitrification

The vitrification technology is now being routinely applied in rapid cooling of stem cells, tissue constructs, and spermatozoa. The primary issue with freezing tissue constructs and a cell sheet on biopolymers is maintaining the integrity and mechanical properties of both the cells and extracellular matrix (ECM) during cooling. Stiffer tissues due to increased cross-linking between

Table 6.7 Vitrification methods for somatic tissue cryopreservation

Tissues	Challenges for cryopreservation	Vitrification methods	Post thaw viability	Limitations of the procedure
Corneal tissue	Endothelium damage	Thin-walled (0.05 mm), Teflon-coated bag	<10 %	*CPA* (*cryoprotective agents*) toxicity; intracellular freezing, which occurs at high cooling rates; and solution effect injury at low cooling rates
Adult stem cells	Loss of undifferentiated state and viability	Open pulled straw	>80 %	Time consuming, labor intensive
Tissue-engineered constructs Combine a scaffold of matrix with living cells to form a tissue-engineered construct (TEC)	CPA needs to permeate entire *TEC* to prevent ice formation	Packaging in a sterile pouch Glass vial	85–95 %	TECs are too large to be vitrified
Chondrocytes/cartilage, primary adult cartilage cells (ACCs)	*CPA* concentration required to prevent ice formation	Glass vial	*Chondrocytes*: 93 % *Cartilage*: 85 %	High thermal processing required to be rapid speed

Table 6.8 Vitrification methods for reproductive cell/tissue cryopreservation

Cells/tissues	Challenges for cryopreservation	Vitrification methods	Post thaw viability	Limitations of the procedure
Human embryonic stem cells	Sensitive to *CPA* (*cryoprotective agents*), requires extremely critical timing and high degree of skill	*OPS* (*open pulled straw*) (1–20 μl)	>75 %	Time consuming, labor intensive
Metaphase 2 oocytes Pronuclear stage Embryos Blastocyst	Extremely sensitive to CPAs	*OPS*, *HSV* (*high security vitrification) straw*, Cryoloop, Cryotop, *EM copper grid* (*EMG*), solid surface vitrification	Zygotes: 90 % survival, 82 % cleavage, 30 % blastocyst	Zona pellucida hardening Alignment of chromosomes is disturbed
Spermatozoa	Sensitive to osmotic shock, large volume	Direct dropping method	50 % decrease in motility	Large sample volume, optimal cooling rate needed
Ovarian cortical tissue	Large specimen size Permeation of the cryoprotectants is challenging	Straws Grids	Pyknosis, vacuolation, cell swelling detected	Thin strips of the cortex are prepared Ice crystallization may occur in the specimen

collagen fibers have been noticed in such cases [50]. Vitrification has been found to be effective in freezing cell population, which present with the above limitations (Tables 6.7 and 6.8).

Vitrification of Stem Cells

Biopreservation of stem cell lines is often perplexing due to the effort in conservation of both differentiated and undifferentiated cells, which

may be in different stages of cell cycle. Cryopreservation offers a fast and reproducible solution for stem cell banking for both stem cell research and clinical use in the field of regenerative medicine.

The pioneering work has come from the field of embryonic stem cell research which dictates that for good post warm survival rates, we must ensure that the embryonic cells used for cryopreservation are morphologically healthy and dynamically proliferating, with minimal differentiation, and should be present in high density.

Open pulled straw (OPS) vitrification has been commonly used wherein human embryonic stem cell (hESC) colonies are dissected and undifferentiated pieces of cellular clumps are placed into sequential vitrification media with increasing concentrations of cryoprotectants. The colonies are now placed into straws and rapidly cooled by plunging them into liquid nitrogen. This method results in a very high (90 %) percentage recovery of the human embryonic stem cell aggregates, with very low percentage of differentiation in the cultures following recovery. Commonly used cryoprotectants are DMSO, ethylene glycol, and sucrose. Care should be exercised while handling DMSO as it can lead to hESC death, apoptosis, and differentiation.

Vitrification has also been successfully used to preserve various others types of stem cells, such as mesenchymal stem cells [51] and hemopoietic stem cells [52]. Post devitrification, cell survival and functionality higher than 80 % were achieved [53] as compared to slow freezing survival rate of 10 % [50].

Besides OPS, both Cryotip and quartz microcapillaries have been assessed for the vitrification of mouse embryonic stem cells. More than 99 % of the cells notably survived after warming without compromising their undifferentiated phenotype expression post vitrification [30, 54].

Since a large number of cells/colonies are to be vitrified when handling stem cells, a high-throughput approach is required. Thus the ejector-based carrier-free droplet-vitrification technique could be a suitable alternative to cryodevices when banking stem cells.

Vitrification of Tissue-Engineered Constructs

Regenerative medicine has shown remarkable prospective potential to repair or replace tissue function that may have been compromised due to illness or injury. The newer strategies presently under expansion include stem cell culture and transplantation along with the use of scaffold materials that generate biochemical signals to stimulate stem cells to initiate differentiation.

Tissue engineering tools have the prospective to develop therapies in the field of regenerative medicine as a potential treatment in the future. The bioreposition of tissue constructs from the corneal epithelia [55], skin [56], oral mucosal epithelia [57], bladder epithelia [58], myocardial cells [59], periodontal ligaments [40], and cartilage [60] is under research. Cryopreserved bioengineered tissue can be used as autografts or allografts and would provide significant boost to clinical applications of cell therapies.

Though the vitrification is an ideal technique for freezing TECs, the technology has its limitations keeping in mind the large size and volume of the tissue constructs which take a longer time to cool and thus raise the chances of cellular toxicity.

Vitrification of Spermatozoa

The initial efforts at cryopreservation of spermatozoa were performed nearly 70 years back. The technology got an impetus after glycerol was added to the freezing media [61]. The motility of thawed spermatozoa normally drops to approximately 50 % post thaw when slow freezing is carried out. Vitrification did not succeed in sperm cooling.

Possibly due to low tolerance of spermatozoa to permeable agents, even a brief exposure to a high concentration of permeable CPAs can lead to toxic and osmotic shock and would be lethal for spermatozoa. It is now proposed to vitrify the sperms in the absence of permeable CPAs using only non-permeable sucrose. The first successful vitrification of human spermatozoa was reported by Isaschenkos' group. Cryodevices like Cryoloops and electron microscope copper grids have been suggested for vitrification of human spermatozoa [33]. Some recommend direct dropping of the sample in LN_2, but this does raise the

issue of the potential risk of microbial or viral cross-contamination. Vitrification yielded the best results with swim-up prepared spermatozoa without cryoprotectant [62].

Vitrification of Ovarian Tissue

Practical understanding concerning cryopreservation of human ovarian tissue by vitrification is presently inadequate. The problems related to successful cryopreservation of ovarian cortex occur due to the complexity of the tissue architecture, which makes the CPA permeation difficult.

The main problems faced are tissue fracturing and ice crystallization during rapid cooling. Both may be avoided by careful handling of the sample during the procedure [63]. To date only a few studies have documented the successful vitrification of human adult ovarian tissue samples [46] using EG and sucrose. Slow cooling has been considered as gold standard for ovarian tissue cryopreservation as compared to vitrification. Few recent studies have compared vitrification and slow programmed freezing of human ovarian tissue. Electron microscopy studies have revealed that the ovarian stroma was significantly better preserved after vitrification as compared to slow freezing. These also did not observe any differences in the ultrastructure of oocytes between non-vitrified and vitrified tissues [64, 65]. Wang et al. revealed in their study that the primordial follicles and stromal cells in vitrified warmed human ovarian tissues by needle immersed technology were better preserved in the vitrified group than the slow freezing [66–68].

Preventing Contamination During Storage

Hermetic sealing of straws and cryovials is an effective measure against contamination of the sample during cryostorage. The use of closed cryodevice carriers for successful storage of vitrified gametes/conceptus reduces risk of viral contamination from liquid nitrogen. Cross-contamination may be prevented by storing infected embryos in separate liquid nitrogen tanks. A preventive step would be filtration of liquid nitrogen and the application of accessory protective storage containers. Sterilization of liquid nitrogen can be carried using sterile polytetrafluoroethylene (PTFE) cartridge filtration [69] or ultraviolet (UV) radiation [70].

Conclusion

Vitrification is a simple, inexpensive, and rapid procedure compared to other cryopreservation methods. The success with vitrification of various stages of cells in reproductive medicine is encouraging the acceptance of this cryopreservation method in ART programs. The development of universal protocols for oocytes, zygotes, embryos, and blastocysts may not be feasible as the response of each cell stage depends on their structure, metabolism, and sensitivity to cryoinjuries. Standardization of definite protocols for each cell stage is preferred.

Existing and emerging applications of vitrification include cryopreservation of stem cells and tissue-engineered constructs. Vitrification exhibits substantial improvement in post thaw cell viability and function compared with the traditional slow freezing methods by eliminating ice-crystal formation. Vitrification using a minimum sample volume enables increased cooling and warming rates and the utilization of lower cryoprotectant agent concentrations, thus reducing toxicity and osmotic shock to cells/tissues. Nowadays it is recommended to use closed devices for rapid cooling, and in the future we foresee rapid emergence of nano- and microscale technologies and droplet-based vitrification techniques to play an important role in the ART laboratories.

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Vitrification of Day 2–3 Human Embryos Using Various Methods

7

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Keywords

Vitrification • Human embryos • Embryos • Cryotec • Embryo cryopreservation

Introduction

Preserving cells at low temperatures is a complex biotechnological process. Its principles were established more than half a century ago, with the progress being driven empirically and most often by trial and error. In assisted reproductive technology (ART), cryopreservation of human oocytes and embryos has been significantly improved by the vitrification technique. Nowadays the slow-cooling systems are completely outdated. The slow-cooling systems have enormous disadvantages when compared with vitrification, such as the requirement of an expensive programmed cryo-machine that usually takes several hours to finish a procedure and prohibitive maintenance costs. Vitrification, on the other hand, has more flexibility; it can be performed at any stage of embryonic development, with little or no fear of malfunction, consistency in performance, and a nearly 100 % success. In the slow-cooling system, it is in addition very difficult to eliminate the injuries resulting from ice crystal formation, and the viability has been reported to be around 40–60 % [1]. Studies have documented the superiority of vitrification over slow freezing owing to the consistent gene expression in human and animal oocytes and embryos achieved by vitrification [1–4].

Vitrification protocols vary widely, and its practice has been heavily dependent on the operator skills, accounting for wide differences in the success rates between embryologists. No single protocol fits all specimen types, and differential vulnerability to cryoinjury remained a major obstacle. As a result vitrification cocktails have proliferated like weeds, some based on painstaking research and others on more homespun formulas. Moreover, cryoprotective agent (CPA) loading, cooling, and unloading have narrow limits of tolerance and should be executed within seconds. Where obsessive attention to detail and green fingers are indispensable, an excellent protocol can be badly compromised in the hands of unpracticed personnel. Since most clinics are also likely to have small patient loads, experience tends to grow slowly and erratically

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[5]. These difficulties have been now overcome by the newer techniques of vitrification.

Despite those experiences, the results of vitrification in terms of survival, pregnancy, and implantation rates were so remarkable that more research and advances were sought and made rapidly in the technology to overcome the obstacles most operators were facing. Within a decade, several advances in vitrification technologies have so tremendously improved clinical efficiency and outcome, reduced the dependence on operator skills, and raised success rates to higher and more uniform levels that the current trend is showing a definite increase in vitrification over slow-cooling techniques. It is for these reasons that vitrification has become a trusted technique.

I remember how Dr. Kuwayama and I were already vitrifying morulae in 1997 with 100 % success when the literature said that was not possible. Since then, our regular success is up to 99 % of survival in embryos at any stage of development and the same results were achieved in oocytes a few years later [6]. However, even for a skilled embryologist, learning the technique from a protocol book is not an easy task. To achieve excellent results, the lab staff needs instruction about critical points of the technique and to understand what they are doing in each step. There are several diverse systems, carriers, and methods available, but Dr. Kuwayama's Cryotop method deserves a special mention. The reason for this is manifold: the technique is effective and yields consistent, easily reproducible results in labs in different countries, not only by our highly experienced hands but also by the skilled hands of every lab personnel. The new and advanced "Cryotec method," which assures one of achieving a 100 % viability in oocytes and embryos at any stage, is set to surely and definitely change the technique of cryopreservation in the entire world.

Developmental Stage on Cryopreservation

It has been observed that cryopreservation of human gametes and embryos has yielded different success rates depending upon the developmental stage of the cell [7]. It is mainly the immature cells which seemed to be more sensitive than those in the later stages, severely restricting its clinical and laboratory applications or procedures. However, today the advances in cryopreservation, particularly in vitrification methods, allow professionals to approach the results as obtained by biological reproduction [8]. The new vitrification methods reach that ultimate goal: 100 % live oocytes, embryos at any stage, and blastocysts.

Different Cryocarriers

During the last decade, numerous carrier systems have been created to allow direct contact with liquid nitrogen or to avoid it, using large or minimal volumes and open or closed systems of the latter, to reduce the theoretical risk of contamination.

The open pulled straw (OPS), Flexipet denuding pipettes (FDP), microdrops, electron microscopic (EM) copper grids, traditional straws, hemistraw system, solid surface vitrification, small nylon coils, and the minimum volume cooling by Cryotips and Cryotops, and recently the open or closed Cryotec (not published yet) are examples of such techniques [6, 9–19] (Table 7.1).

In 2005, Kuwayama et al. reported improved vitrification success with the use of Cryotop in human oocytes and also published a comparison between the open system, the Cryotop, and a closed vitrification system, the Cryotip, over 13,000 embryos at different stages. This is the largest study to date on vitrification in which the authors have suggested that Cryotop is an efficient and reliable method to freeze cleaved embryos, blastocysts, and oocytes in daily practice. The impressive clinical success rates at the Kato Ladies Clinic and other centers in the past decade have encouraged clinics around the globe to switch to vitrification for oocytes and embryos.

The idea of transmission of viral pathogens to vitrified and stored embryos in contaminated nitrogen was raised by Bielanski et al. [21]. However, cross contamination of these agents and

Table 7.1 Different carrier systems

Method	Reference
Electron microscopic grids	Martino et al. (1996) [9]
Open pulled straw (OPS)	Vaita et al. (1998) [10]
Glass micropipettes (GMP)	Kong et al. (2000) [11]
Gel loading tips	Tominaga and Hamada (2001) [12]
Closed pulled straw (CPS)	Chen et al. (2001) [13]
Nylon mesh	Matsumoto et al. (2001) [14]
Sterile stripper tips	Kuleshova and Lopata (2002) [15]
Flexipet denuding pipette (FDP)	Liebermann and Tucker (2002) [16]
Cryoloop	Mukaida et al. (2003) [17]
Cryotip	Kuwayama et al. (2005) [18]
Cryotop	Kuwayama et al. (2005) [6]
Cryoleaf	Huang et al. (2005) [19]
Cryotec	Gandhi et al. (2014) [20]

transmission between samples have not occurred in human ART in 500,000 vitrified embryo and 140,000 vitrified oocytes. Of all these carriers, Cryotec is now being reported as an open or close device which could eliminate the theoretical risk of contamination of cells while maintaining an astonishing efficacy.

Economical Evaluation of Vitrification

Vitrification technique takes a total time of about 10–15 min that is nearly ten times less than the time required for slow cooling. Furthermore slow cooling is quite an expensive method when compared to vitrification in terms of equipment and running costs. In 2005, it has been stated that the primary disadvantages of slow cooling in cryopreservation of human embryos are the requirement of an expensive programmable freezing machine and that the process is time consuming [6, 18, 17, 22]. Vitrification on the other hand can be performed without the use of costly equipments and can be completed by only one specialist within minutes. Hence, the introduction of vitrification has decidedly been able to provide significant benefits to any busy IVF lab.

Vitrification of Human Zygotes and Cleaved Embryos

In 1985, Rall and Fahy were the first to report the vitrification method in mouse embryo cryopreservation, but it was not applied in humans [23]. In 1998 Mukaida et al. reported successful vitrification of human 4–8 cell embryos using the method developed for mouse embryos, and other groups also confirmed that vitrification is indeed applicable to human embryos reporting that pronuclear stage embryos could survive with high rates after vitrification and warming procedures [6, 24]. Between 2001 and 2010, different survival rates were reported mainly because of the variation of vitrification solutions, protocols, and the embryologist skills [16, 25, 26].

However those studies in vitrification of early-stage embryos reported survival rates up to 80 % and pregnancy rates of 22–30 %, higher than the rates obtained by slow rate freezing procedures [6, 16, 25–35] (Table 7.2).

Today it is very clear why a blastocyst can be more relied upon to produce pregnancy. This is due to the fact that blastocysts are better suited to the uterine environment than previous stages and the blastocyst formation selects for more viable outcome [36]. But for laboratories in which it is not yet possible to produce blastocysts, vitrification at the early embryo stage is still a viable option. Acceptably high rates of pregnancy have been reported using vitrification at even the cleavage and pronuclear stage. Vitrification of early-stage human embryos is an effective, viable, and superior alternative to slow rate freezing, with increased rates of survival and pregnancy, which is essential for centers using routine day 3 transfers or those performing preimplantation genetic diagnosis procedures. Vitrification at this stage will serve very well in such countries where further culturing is not allowed.

In vitro culture systems evolved with sequential media, and it is now quite easy to develop human IVF embryos into blastocysts, culturing embryos until day 5 [37]. Extending the embryo culture program and transferring a blastocyst on day 5 increased implantation rates to 32 % in comparison to day 3 transfer (23 %) with a

Table 7.2 Vitrification of early-stage embryos

Year	Authors	Stage	CPA	Carrier	Vitrified embryo	Survival rate (%)	Pregnancy rate (%)
2012	Wang et al. [28]	6–8 cell	EG/DMSO/S	Cryoleaf	825	86.6	36.3
2009	Rama Raju et al. [29]	6–8 cell	EG based	Cryoloop	907	90.37	36.84
2008	Balaban et al. [30]	6–8 cell	EG/PE	Cryoloop	234	94.8	30
2007	Al-Hasani et al. [31]	Zygote	EG/DMSO/S	Cryotop	339	98	36.8
2007	Desai et al. [32]	6–8 cell	EG based	Cryoloop	90	85	
2005	Kuwayama et al. (2005) [18]	PN	EG/DMSO/S	Cryotop	13,000	100	27
		4 cell		Cryotop		98	
2005	Zhu et al. [33]	Embryo	EG based	OPS	957	72.2	22
2005	Rama Raju et al. [34]	Embryo	EG/S	OPS	40	95	35
2005	Hredzak et al. [27]	Cleavage	EG based	Pipetting tip	215	69	27
2003	Isachenko et al. [35]	PN	EG based	OPS	59	71	NA
2002	Liebermann and Tucker [16]	Embryo	EG based	FDP	266	83.8	NA
		Oocytes	EG based	FDP	928	80.6	NA
2001	El-Danasouri and Selman [26]	Embryo	EG based	OPS	215	49.3	30.5
2000	Saito et al. [25]	Embryo	EG/Ficl/HTF	OPS	98	100	16.2

CPA cryoprotective agent, *DMSO* dimethyl sulfoxide, *EG* ethylene glycol, *S* sucrose, *FDP* Flexipet denuding pipette, *HTF* human tubal fluid, *OPS* open pulled straw, *PE* propanediol, *NA* not available

significant increase in pregnancy rates in addition to a significant decrease in the number of embryos transferred [38].

Conclusion

Cryopreservation has become a mainstay of the assisted reproduction laboratory and supports fertility preservation program for patients with cancer and other indications. Vitrification is steadily replacing the former slow freezing protocols because of improved survival rates and clinical outcomes.

Vajta and Nagy stated in 2006: “Papers unanimously support the application of vitrification and point out that this is the future of cryopreservation. The public have the final say in whether they want and allow this future to arrive” [39]. We are definitely in that future now, and the situation has changed completely since 2006. We have successfully vitrified half a million embryos and 140,000 vitrified oocytes are waiting for IVF, with no evidence of contamination, giving the same results as fresh oocytes and embryos, in more than 1,000 IVF centers in 40 countries (accumulated data from ART clinics around the world) using these low-cost and easy-to-perform vitrification methods.

There is no requirement of special machines and special conditions for introducing vitrification as a routine protocol to an ordinary IVF laboratory. Only standard laboratory devices, storage tanks, and training of the embryologists, which is quite easy for a standard IVF laboratory team, are necessary for this purpose. Therefore, any laboratory can convert to routine vitrification within a few days without any specific setup [40].

Minimal volume methods, in particular Cryotec method, are the most efficient in terms of survival (100 %), embryo quality, implantation rates, and clinical pregnancy. Results are comparable to using fresh oocytes or embryos with regard to survival, fertilization rates, and embryonic development. The method is also suitable for patients who wish to delay motherhood and patients in reproductive age with cancer. With the huge amount of data from groups around the world and the large experience acquired, we can affirm that vitrification is safe, decidedly effective, repeatable, and easily applicable in varied clinical circumstances, therefore justifying its application as a routine technique in the treatment of infertility and preservation of fertility.

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8 Current Trends in Fertility Preservation Through Egg Banking

Pratik Tambe and Goral Gandhi

Keywords
Fertility preservation • Oocyte vitrification • Elective oocyte cryopreservation

Introduction

Embryo cryopreservation is today the most established method of fertility preservation [1]. Oocyte cryopreservation now offers a new option for single women in the reproductive-age group in need of delaying childbearing for any reason. Due to practical problems related to the structure of the oocyte and optimization of freezing methods, it has taken more than 20 years for oocyte cryopreservation to evolve into a technique with acceptable clinical pregnancy rates.

This transition was made possible by three important achievements: utilization of intracytoplasmic sperm injection (ICSI), improvements in cryoprotectants, and introduction of vitrification [2–5]. The improvements in the technique and the recent removal of the "experimental" label on oocyte cryopreservation by the American Society of Reproductive Medicine (ASRM) Practice Guideline Committee [6] have ushered in a new era.

Oocyte cryopreservation is expected to take the lead in fertility preservation. It is also likely to become a useful adjunct to routine IVF in various clinical scenarios such as the unavailability of sperm at the time of egg retrival [7, 8], in cases of ovarian hyperstimulation syndrome [9], in poor responders [10, 11], in patients at risk of losing their fertility potential due to genetic abnormalities such as BRCA mutation carrier status [12], Turner syndrome [13], fragile X syndrome, and deletions of the X chromosome [6] and for couples who do not wish to cryopreserve supernumerary embryos for ethical, legal, or religious concerns [14].

Another indication for oocyte vitrification that has now become a reality [15, 16] is the establishment of donor oocyte banks. In future, IVF cycles using frozen-thawed donor oocytes may outnumber those using fresh donor oocytes. However, elective oocyte cryopreservation (EOC) for deferring childbearing remains the

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most controversial but, surprisingly, the most common indication for oocyte cryopreservation. Most centers currently performing oocyte vitrification in the United States do so for elective indications [17].

There are many factors that affect the efficiency of success with oocyte cryopreservation such as factors related to host (age, donor/nondonor oocyte, infertility factor), stimulation protocols, cryopreservation methods (slow freezing and vitrification), protocols, and devices (cryotop, cryoleaf, cryotip). Hence, it is difficult to reliably estimate the success of oocyte cryopreservation from various studies. The majority of the studies on oocyte cryopreservation are observational, and only six RCTs with clinical outcomes have been published [15, 18–22].

History

The first live birth with oocyte cryopreservation was reported in 1986 with slow freezing [23]; but due to very low success rates, there were only five live births reported initially [24]. In 1997, intracytoplasmic sperm injection (ICSI) was first used to fertilize frozen-thawed oocytes, circumventing zona hardening caused by the cryopreservation process [25]. Further optimization of oocyte cryopreservation required another decade. In 1999, the first live birth with oocyte cryopreservation after vitrification was reported [26] followed by a few case reports until 2005 [24].

There were only 100 reported live births from oocyte cryopreservation. A meta-analysis [24] of these concluded that success rates with oocyte cryopreservation using slow freezing were lower than that of IVF with fresh oocytes. Comparisons of vitrification with either slow freezing or fresh oocyte cycles could not be performed because of the limited number of reports with vitrification at the time of publication. However, the success rates with vitrification showed encouraging results compared with slow freezing.

Following the first RCT comparing slow freezing and vitrification, which showed that vitrification was more successful in terms of both embryological and clinical outcomes [18], more workers reported improved clinical outcomes using vitrification [14–16, 19, 27–31]. Efficiency of slow-freezing protocols has since improved [32–40]. However, success rates remain lower for slow freezing compared with vitrification.

With the improvements in oocyte cryopreservation technology and associated clinical outcomes, its clinical applications widened, with more than a thousand live births reported to date [17, 41]. Over the past 5 years, oocyte cryopreservation, especially with vitrification, has proven to be an efficient technique, resulting in pregnancy outcomes similar to that of IVF with fresh oocytes [15].

A recent RCT has shown that aneuploidy rates in embryos derived from vitrified oocytes were similar to those from fresh oocytes in young infertile women undergoing IVF with their own eggs [21]. This suggests that oocyte cryopreservation does not have an adverse effect on chromosome segregation during meiotic division.

Clinical Application

Research into IVF outcome parameters using oocyte cryopreservation falls into one of two main categories: (1) studies assessing donor oocyte cryopreservation/thaw cycles representing young fertile women and (2) studies assessing infertile women with failed IVF attempts, who have supernumerary oocytes for cryopreservation. Studies on oocyte cryopreservation for poor responders [11] and for IVF cycles with failed sperm retrieval [8] constitute the exceptions.

Nondonor or Autologous Oocyte Cryopreservation

Randomized Controlled Trials

Most reports on cryopreservation of nondonor oocytes are observational studies, with only a few RCTs performed in infertile women undergoing IVF who prefer cryopreservation of their surplus oocytes. These women declined embryo

cryopreservation due to ethical or legal concerns. Also, there are studies that compare the efficacy of oocyte cryopreservation by temporarily cryopreserving oocytes under an institutional review board approval [21]. There is only one single study to date assessing the efficiency of nondonor oocyte cryopreservation when applied to young fertile women [42].

There are four published RCTs on nondonor oocyte crypreservation; all four report the outcomes of IVF using vitrified/warmed nondonor oocytes from infertile patients [18–21]. At present, there are no RCTs evaluating IVF outcomes of slow frozen oocytes compared with fresh oocytes. Only one study compared slow freezing and vitrification, reaching the conclusion that vitrification is superior to slow freezing in terms of oocyte survival, fertilization, implantation, and clinical pregnancy rates. This is the only RCT comparing the two techniques [18].

Two RCTs were conducted in infertile couples with supernumerary oocytes available to vitrify and warm only if pregnancy was not achieved in the fresh cycle [19, 20]. Fresh sibling oocytes were transferred in the first cycle. If pregnancy failed to occur, then the cryopreserved sibling oocytes were thawed, fertilized, and transferred to the same patient in a subsequent cycle. Using this design, the authors were able to compare the fertilization and embryo developmental rates of vitrified and fresh sibling oocytes. Both studies concluded that similar fertilization and embryo development rates were achieved with fresh and vitrified oocytes [19, 20].

In the most recently published RCT, Forman et al. [21] adopted a unique design, which allowed the comparison of clinical outcomes with nondonor vitrified and fresh oocytes. In this study, the authors divided retrieved oocytes from infertile patients less than 35 years of age. One group of oocytes underwent temporary vitrification while their others remained in culture. Later, vitrified oocytes were thawed; vitrified and nonvitrified oocytes were fertilized with ICSI, and resulting embryos were cultured to the blastocyst stage.

Embryos of sufficient quality to transfer or cryopreserve underwent trophectoderm biopsy for genotyping and a karyotype was assigned to each embryo. Blastocysts obtained from vitrified and fresh oocytes were then transferred in pairs, and embryonic aneuploidy was assessed in each one. To determine the identity of the implanted embryos, DNA fingerprinting was performed on cell-free fetal DNA enriched from maternal serum specimens drawn at 9 weeks of gestation or on newborn DNA taken from a buccal swab.

The authors detected no differences between the two groups regarding aneuploidy. In addition, the ongoing pregnancy rate per transferred embryo was similar for vitrified and fresh oocytes. However, the fertilization and embryo development rates were lower in vitrified compared with fresh oocytes. This finding is in contrast with previous trials reporting similar fertilization and embryo development rates for both nondonor [19, 20] and donor cryopreserved oocytes [15, 22] compared with fresh oocytes.

Importantly, oocyte vitrification does not seem to increase the rate of aneuploidy or diminish the implantation potential of viable blastocysts. The authors demonstrate that clinical success rates with nondonor vitrified oocytes from young infertile women are similar to their sibling fresh oocytes.

Overall, RCTs investigating the use of cryopreserved nondonor oocytes from infertile patients suggest that vitrification is more successful compared with slow freezing [18]; fertilization and embryo development rates of vitrified oocytes are comparable to fresh oocytes [19, 20], and for women less than 35 years, pregnancy rates and embryo aneuploidy rates of vitrified oocytes are similar to fresh oocytes [21].

Observational Studies

Many observational studies on the efficacy of oocyte cryopreservation have been reported, and most of these studies (almost 90 % of slow freezing and 50 % of vitrification studies to date) were conducted in centers located in Italy. This is because Italian law prohibits insemination of more than three oocytes and has banned embryo cryopreservation, which has forced oocyte cryopreservation into routine clinical practice.

In the largest of these studies, supernumerary oocytes from infertile women were cryopreserved using slow freezing, and 940 thaw cycles were performed in eight centers [38]. The overall survival rate of thawed oocytes was 55.8 %. The fertilization rate (72.5 vs. 78.3 %), implantation rate (10.1 vs. 15.4 %), pregnancy rate per transfer (17 vs. 27.9 %), and delivery rate per transfer (11.6 vs. 21.6 %) were all significantly lower for cryopreserved oocyte cycles compared with fresh cycles.

Despite the reported lower success rates, the protocol is still evolving [39]. Recently, Azambuja et al. [40] using a sodium-depleted media and Bianchi et al. [39] using a modified slow-freezing protocol reported higher encouraging success rates with slow freezing.

Novel Indications

Although oocyte cryopreservation is proposed for preserving fertility in cancer patients, the data on clinical success of oocyte cryopreservation in such patients are limited. For the purposes of counseling, success rates might be extrapolated from other populations.

Cancer patients are treated with the assumption that their reproductive potential is similar to that of age-matched healthy individuals.

Some studies suggest comparable results with nondonor patients [43–46], whereas others show diminished oocyte yield [47–50]. If further studies with larger sample size confirm that women with cancer have diminished ovarian reserve, appropriate counselling of these women is crucial as women with diminished ovarian reserve are expected to be more susceptible to gonadotoxic agents.

Oocyte cryopreservation has been used in poor responders [11] and in situations when sperm cannot be obtained for IVF [7, 8]. Cobo et al. [11] have reported a new strategy with vitrification for managing poor responder patients. They have proposed that for poor responders, accumulation of oocytes by vitrification and simultaneous insemination yields live birth rates comparable to those in normoresponders.

Donor Oocyte Cryopreservation

There are two RCTs using vitrified donor oocytes [15, 22]. The largest RCT including 600 recipients of donor oocytes demonstrated similar ongoing pregnancy rates with vitrified donor oocytes when compared with fresh donor oocytes [15]. This study reported implantation and clinical pregnancy per embryo transfer rates of 39.9 vs. 40.9 % and 55.4 vs. 55.6 % for vitrified donor and fresh donor oocytes, respectively.

Oocyte donors are women under the age of 35; therefore, the results of these studies may be extrapolated to young patients seeking fertility preservation. With the current excellent reported success with cryopreserved donor oocytes, it is now possible to justify and establish oocyte cryobanking. This strategy will also allow quarantine of oocytes.

Trends in Nondonor Oocyte Cryopreservation

There are numerous nonrandomized studies investigating IVF outcome parameters associated with oocyte cryopreservation. As there are differences between these studies in design, cryopreservation protocols, indications for cryopreservation, age of the patients, number of oocytes thawed and embryos transferred, it is not appropriate to compare the success between different studies. However, these studies show that while the clinical success rates with slow freezing have an increasing trend with time, vitrification has been more successful than slow freezing.

Since 2006, implantation and live birth rates increased from 2 to 14 % and 2 to 27 % [39, 40, 51] for slow freezing, while they ranged from 13 to 20 % and 23 to 35 %, respectively, following a more closer trend for vitrification [19, 20, 29, 52].

Age Trends

Most of the studies published to date reported results according to the mean ages of the patients,

which range from 29.9±2.3 [21] and 35.7±5.7 [36]. However, it is not appropriate to use the reported success rates when counselling patients individually, as the success of IVF using cryopreserved oocytes is likely to be affected by the patient's age.

According to an individual patient data meta-analysis, live birth success rates with cryopreserved oocytes show an age-related decline regardless of the freezing technique used, and an aged-based probability of live birth may be calculated for cryopreserved oocytes [53].

Estimated age-based success rates may also change according to the indication for oocyte cryopreservation, such as elective oocyte cryopreservation or oocyte cryopreservation in poor responders. For example, in poor responders, accumulating cryopreserved oocytes in consecutive cycles followed by thaw, ICSI, and embryo transfer is reported to yield comparable success rates to those observed in normal responders [10, 11].

When this strategy was applied to poor responders over 40, live birth/patient success rates were higher (15.8 %) for the vitrified oocyte group compared with the fresh oocyte group (7.1 %) [11]. Recently Melzer et al. [54] reported a similar approach for patients undergoing EOC. In that study of 132 patients undergoing multiple cycles of EOC with an average age of 38.4 at first and 39 at subsequent cycles, when more than one cycle was applied, subsequent cycles resulted in greater oocyte yield, albeit with the implementation of a higher dose.

Elective Oocyte Cryopreservation

Among the programs in the USA offering EOC, half accepted women aged 38–40 years, and about one-third accepted women above 40 years [17]. A recent study analyzing 491 women reported that mean age of the patients undergoing EOC was 38 [55] in accordance with two other studies [56, 57]. Importantly, more than 80 % of women undergoing EOC were over 35 years old (range: 36–41) [56].

Despite the reported interest of older reproductive-age women toward oocyte cryopeservation [55–57], one of these studies found that the mean age of women inquiring about this procedure was 35.2±5.4 years [57]. The same study reported that the age for the application of EOC decreased from 39+1.4 years in 2005 to 37.4+2.3 years in 2011.

This shows that EOC is primarily utilized by older reproductive-age women, although women inquire about the procedure earlier. However, to achieve higher success rates with IVF, both the age of inquiry and application of EOC should be at an age younger than 35 years [58].

Conclusion

Following the first live birth with cryopreserved oocytes in 1986 and a very slow progress for 20 years, clinical outcomes using cryopreserved oocytes have made great strides during the past decade. Recent RCTs show that fertilization, embryo development, and pregnancy rates with vitrified nondonor and donor oocytes are similar to fresh oocytes. Vitrification remains the protocol of choice as the overall success rates with slow freezing remain lower compared with vitrification.

These improvements in the cryopreservation technique and clinical outcomes may result in an increased utilization of oocyte vitrification in clinical practice. In order to provide appropriate counselling to women considering oocyte cryopreservation for fertility preservation or as an elective procedure for deferring child bearing, it is necessary to arrive at age-specific and indication-specific success rates so that we may better inform our patients.

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9 Nitrogen Vapor Shipment of Vitrified Cells: Challenges, Caution, and Emerging Opportunities

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Keywords

Transport • Dry shipper • Liquid nitrogen • Vitrification • Vapor shipment

Introduction

Assisted reproductive technologies (ART) have evolved due to the ability to cryopreserve human gametes and embryos and subsequently thaw them with predictable results. There is enormous data in the literature that clinical outcomes with cryopreserved gametes/embryos are same as the ones with fresh samples [1–3]. Cryopreserved specimens are routinely stored in liquid nitrogen.

Short-term vapor storage for transport as well as long-term storage of cryopreserved samples in the LN2 vapor phase has been an area of interest. Traditionally, LN2 dry shippers have been routinely used for shipping slow frozen embryos and gametes. Shipping of cryopreserved specimens between assisted conception units might have subsequent effect on viability and development, where specimens are expected to experience the multiple ambient exposures required to move the cryounits between tanks and shippers. There is further possibility of more dramatic negative effect of shipping on vitrified specimens, where the volumes are small (<1 μl).

The movement of cryopreserved specimen is now routinely carried out between facilities with good outcomes [4, 5], although some recent studies have revealed that problems can occur albeit with a low incidence [6, 7].

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Cryoshipping

Cryopreservation by immersing and storing the sample in liquid nitrogen (−196 °C) has been the standard method of cell storage. These cryopreserved specimens appear to be quite stable over time once in storage. However, cryopreserved specimens are occasionally required to be transported to another assisted conception. However, transportation of embryos in liquid nitrogen is costly as well as more risky. If the container is not kept upright, the liquid nitrogen may spill out and compromise the cells due to increase in the container temperature. Nowadays, transfer of cryopreserved human gametes and embryos employing "dry" nitrogen vapor shipping tanks is a common practice worldwide. Specifically

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designed dry shippers are often used successfully [4, 5] and are accepted as safe by the International Air Transport Association [8].

Dry Vapor Shipper

The dry shipper allows the LN2 to be fully absorbed in a porous foam retention medium that surrounds the cryoshipper chamber. Cold vapor emanating from the liquid nitrogen, entrapped within the foam retention system, provides validated cooling, thereby eliminating the need for recycling on long-haul and cross-border shipping lanes. The foam retention system fully absorbs the LN2, preventing spilling, regardless of orientation, and yet keeps the vessel only a few degrees warmer than liquid nitrogen itself. The excess liquid is removed from the container prior to shipment. A vapor container is not considered a hazardous item and may even be placed on commercial passenger airlines for transport. A vapor shipping container can maintain temperature below −150 °C for a 10+-day dynamic shipment if it is charged to its maximum limit (see Fig. 9.1).

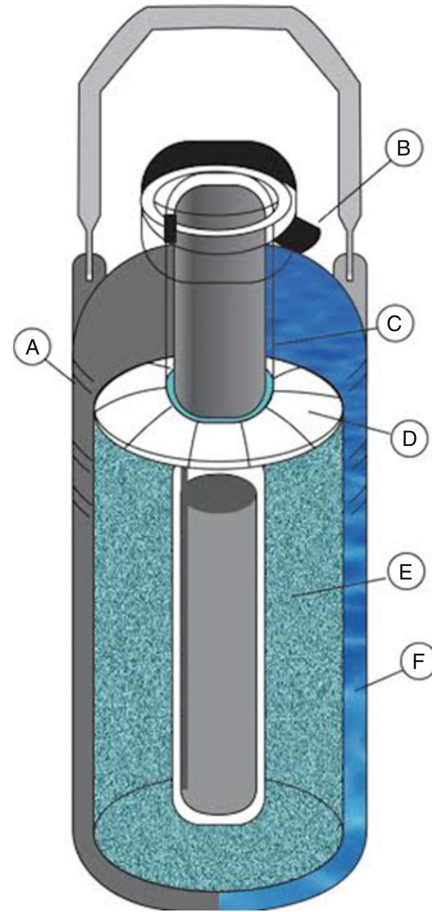

Fig. 9.1 Liquid nitrogen vapor shipment. (*A*) Lightweight aluminum design. (*B*) Locking tab. (*C*) Neck tube. (*D*) Chemical vacuum retention system. (*E*) Hydrophobic absorbent. (*F*) Insulation

Challenges

Problems during transport have been described in the literature despite specifically designed dry shippers, including failure of the dry shipper [7] and poorer outcomes using the transported material [6].

Effect of Vapor Phase Storage

An important consideration while using nitrogen vapor shipment is the ability of cryopreserved specimen to sustain viability when held only in the vapor phase during transportation. Dry shippers for transporting are designed to keep the sample at −150 °C in the vapor phase of LN2. As the temperature in the vapor phase of liquid nitrogen is higher than that in liquid nitrogen, it may affect the quality of the cryopreserved specimen. Vapor storage has been successfully applied to cryopreserved sperm [9, 10], oocytes [4, 11], and embryos [12].

Tomlinson and Sakkas found no difference in the survival of sperms whether stored in liquid or vapor nitrogen [13]. Eum et al. evaluated the effect of long-term storage of rapid and slow cooled mouse embryos [12]. Embryos were warmed at intervals of one week, one month, and six months. Apoptosis scores and continued development post warming at each interval suggested that storage of both vitrified and slow frozen embryos in either liquid or nitrogen vapor was not different.

The effect of vapor phase storage on embryonic development and DNA integrity in vitrified

embryos was examined by AbdelHafez et al. [14] Based on the outcome parameters, holding vitrified embryos in the vapor phase of LN2 did not result in obvious impairment of development or a significant increase in damage to cellular DNA. This finding is reassuring, suggesting that transport of vitrified embryos may be possible with minimal harm.

Recently, Chang and colleagues reported high survival rate (85.7 % vs. 87.0 %) in human oocytes vitrified in open system and exposed in dry shipper for 60 h [4]. The survival rate of vitrified oocytes was not affected after exposure to the environment of the dry shipper. These results suggest that oocytes cryopreserved by the vitrification technique may be shipped safely using the dry shipper.

All of the studies concluded that vapor phase storage was as efficient as liquid phase storage, when stored at temperature well above glass transition temperature of −130°C. Vapor phase storage has also been proposed as a means to circumvent the risk of sample cross-contamination, especially where open vitrification carriers are stored directly in LN2 [15].

Effect of Method and Carrier of Freezing

The traditional slow freeze technique is most often performed using a "closed" system without direct liquid nitrogen (LN2) exposure in the straws and vials that utilize large volume, (0.25–0.5 ml) in straws and (1 or 2 ml) in vials. In comparison most vitrification carriers utilize microdrops <1 μl in order to achieve high cooling rates. The effectiveness of different carriers for transporting embryos during vapor phase storage may vary. Open carriers allow direct contact of embryos with LN2, whereas closed carrier systems sequester the embryo within a sealed system during immersion in LN2 avoiding any contact between cell/tissue and LN2. Vitrified embryos in closed carriers are completely sequestered from the LN2 and are therefore potentially more vulnerable to temperature variations, especially when stored in vapor phase versus directly in the LN2 phase.

Reed in his elegant study compared the efficacy of shipment of embryos between clinics in liquid nitrogen vapor using various freezing techniques – Slow cooling method using CBS straws, vitrification method using HSV CBS straw, Fibreplug, and CryoLoop [16]. HSV CBS straw is a closed system vitrification carrier, whereas Fibreplug and CryoLoop are open system vitrification carrier devices. Each of the four cryopreservation treatments exhibited similar outcomes despite the volume differences. There was no effect of concomitant ambient exposures on post-warming embryonic development, in either slow or rapid cooling treatments; however, there did seem a negative developmental trend (a shift away from complete hatching from the zona pellucida). He concluded that the results are encouraging, with regard to the use of vitrification techniques and shipping the gametes or embryos between clinics in nitrogen vapor shipment.

Thermal Exposure

The most important consideration with the process of shipping involves brief exposure to air as the cryopreserved specimens are transferred between different cryostorage containers. As the process of shipping involves brief exposure to air while being transferred between different cryostorage containers, there is differential susceptibility depending on the method and carrier of freezing. The exposure time using the "8- to 10-second rule" as described by Stroud, in his excellent cautionary article on handling frozen gametes and embryos, may be considerably too long for very small volumes utilized in vitrification [17]. Poor handling of cryopreserved samples can impact their post-thaw survival and viability [17]. With specifically designed shipper, there is less concern regarding the core temperature of properly charged shipper. The major concern is the number of, and/or duration of, ambient temperature exposures during movement of specimens from the storage environment to a shipper and back to the storage after transport. Cellular damage occurs when the internal temperature rises above −130 °C and then reintroduced to

below those temperatures [17]. Rapatz reported that although some cell damage can occur at −130 °C, ice is relatively stable at – 100 °C but becomes more vulnerable at −80 °C [18]. Since the temperature in the necks of most standard dewars ranges from – 75 °C to room temperature, it is very common for frozen semen and embryos to be exposed and damaged, or even destroyed, during routine handling. A temperature-specific dye could be supplemented to frozen samples that would react if the internal temperature of the straw or vial of the frozen sample goes above −130 °C.

Shipping Hazards

In the real world, the shipment may encounter variables such as mishandling of the shipper, tipping of the tank, long transit time, multiple personnel handling, and samples remaining in shipper for a day or more after delivery to the destination clinic. There is an inherit inability to constantly analyze the nitrogen vapor shipping container during transportation. Besides being fully charged and reading −196 °C upon departure, any additional conditions to which the containers may have been subjected are beyond ability to prevent or observe. Inherent perils of shipping apply, for example, being bumped, dropped, or stored in the wrong orientation, ambient temperature, and air pressure. The increased sensitivity of vitrified material to unavoidable shipping hazards is a reason for caution.

Technical Aspects

Vitrified cells do pose a challenge to technicians in the field. The embryologist from the clinic receiving the frozen samples should be skilled enough to perform the thawing process by the method used to vitrify the cells. It is also necessary for the embryologist to understand the paperwork forwarded by the previous clinic and interpret it. Heat dissipation occurs much faster in vitrified specimens as they have small surface area. As a consequence, anyone handling vitrified cells should be extremely cautious when manipulating frozen samples in the neck of vapor dewars.

Caution

Transporting cryopreserved cells to and from a long-term storage facility or to other laboratory may have several adverse effects on cryopreserved cells. Extra caution should be practiced, especially when handling and transferring cryopreserved specimens between shipping and long-term cryopreservation storage containers.

At some point after having been charged with liquid nitrogen, a dry shipper will begin to warm. The internal temperature of the cryoshipper can be warmed from −190 °C immediately post charging to −138.9 °C 10 days later [17], that is, getting very close to the glass transition temperature of water, the temperature where ice becomes unstable and recrystallization begins to occur. Various measures have to be taken at different steps, which may be necessary to minimize exposure of samples to temperature changes.

Caution and closer examination of vitrification methods is required to ensure that hypersensitive tissue in microvolumes are adequately protected and properly handled. This would include extensive testing of the different types of storage devices (i.e., tips, loops, leaves, tops, straws, and vials), as well as a comparison of the closed and open systems.

Personnel Know-How

Poor handling habits by a technician during cryoshipping can result in cumulative damage that can decrease the pregnancy rates and in some cases lead to total infertility. These mistakes often lead to extensive and grueling hunts by clinicians and embryologists trying to diagnose the reason behind low conception rates using frozen samples.

Most assisted reproductive laboratory personnel are familiar with straws or vials for slow freezing, but far less familiar with the myriad of vitrification methods in use. In order to achieve

best outcomes, both the concerned labs need to communicate and the lab personnel involved with the movement should be familiar with the procedure and protocol of vitrification.

Emerging Opportunities

Vapor shipment is of much use when considering import/export of human gametes from different countries. Travelling to a new country for fertility treatment is time consuming for the patients. In addition it involves a lot of paperwork as legal regulations regarding ART vary from country to county. It is challenging for the patients to keep up with the necessary requirements given the emotional turmoil they find themselves in owing to infertility.

Fertility Tourism

Fertility tourism is a form of medical tourism in which the patient travels to another country for fertility treatments. The most common reasons for fertility tourism are as follows:

Financial

The primary reason is due to financial problems, many a times the required treatment is cheaper in other countries.

Legal

Legal regulation of the sought procedure in the home country for example, clients from Britain often travel to the United States in order to undergo preimplantation genetic diagnosis (PGD) for determining the sex of the fetus, since the same is illegal in their own country [19].

Most people go to foreign countries for egg donation or surrogacy cycles. Egg donation is banned in a number of European countries including Germany, Austria, and Italy; hence, the patients travel to other countries like the United States or Spain where the laws are much more relaxed. India is a leading destination for surrogacy, because it is comparatively less expensive as compared to the other countries [20].

The United Kingdom allows the children of the donors to know the identity of their biological parents; hence, the country is facing shortage of sperm and egg donors as they are not willing to disclose their identity [21]. As a result, people are obtaining gametes from other countries.

Availability of the IVF procedure with less restriction on age, weight, marital status, and sexual orientation may drive patients for fertility tourism.

Surrogacy and Repeated Attempts

Also, for patients opting for surrogacy or repeated attempts, it is not feasible to travel each time to their desired clinic in a particular country. For such conditions, the frozen samples can be transported for their future use.

Advances in Technology

Fertility tourism is often used for seeking specific fertility treatments that involve techniques, which may not be available in their home country.

According to a study carried out by the European Society of Human Reproduction and Embryology (ESHRE) in 2009, annually, around 20,000–25,000 cross-border fertility treatments are carried out with Israel being the most preferred fertility tourism destination in the world having the highest number of fertility clinics per capita [22].

Cryoshipping plays an important role particularly in IVF cycles involving use of donor gametes, as it is cumbersome if travel of donors along with patients is required. Hence, in current scenario of fertility tourism and also donor programs, vapor shipment holds an important place. Also, donor banks are now a separate entity and belong to third-party reproduction which is different from the destination clinic where these samples are required.

Rotunda: CHR Cryoshipping Program

Rotunda has received cryoshipped embryos from across the globe. The samples were frozen with a myriad of vitrification methods and carrier devices. Specimens were received from over 15 countries, spanning five continents (see Fig. 9.2).

Fig. 9.2 Rotunda cryotanks and cryoshippers

International Shipments

The very first step in any international shipment is to contact consignee in order to determine which documents are needed to get a clear certificate from the customs. An airway bill and commercial invoice are required for an international shipment. Other documents may include an import permit, a declaration letter, or certificate of origin.

The commercial invoice should have the shipper's name and address, the consignee's name and address, the value of the shipment, and the nature and quantity of the items being shipped. The commercial invoice must be signed by the shipper. Depending on personal choice, insurance can be taken for biologic material (see Fig. 9.3a, b).

Handling of Shipper

All storage and shipping vessels are not the same, and care must be taken to limit the physical position of the shipped specimen to the central lowest part of the tank [17]. Movement of the cryounits from storage to shipping unit and the reverse upon arrival after shipping must be done as quickly as possible with as little ambient exposure as possible. Once the dry shipper has left the laboratory, several things can go awry.

The list of potential wrongdoers is long: lab technicians, courier personnel, and embryologists. Very few, if any, courier personnel have ever been trained in proper handling techniques for frozen biologicals. There are several times when exposure is most likely to happen. The following is a list of some of those opportunities:

1. Taking inventory and making proper paper work
2. Preparing samples for shipment
3. Receiving and transferring samples from a dry shipper to a storage tank
4. Thawing process

When a shipping request is made, a skilled technician should properly charge a dry cryoshipper, retrieve the frozen sample, and quickly move it into the shipper. Utmost care must be taken while transferring the canes of sample from a large storage tank, at no point will the technician allow the frozen straws to reach temperatures above −130 °C (see Fig. 9.4).

As soon as the dry shipper reaches its destination clinic, contents should be removed immediately from the dry shipper and placed in liquid nitrogen storage tank. Be aware that as the Styrofoam lid from a dry shipper is lifted, vapor

Fig. 9.3 (**a**, **b**) Cryoshipper MVE SC 4/3 V: Vapor shipper with protective cover

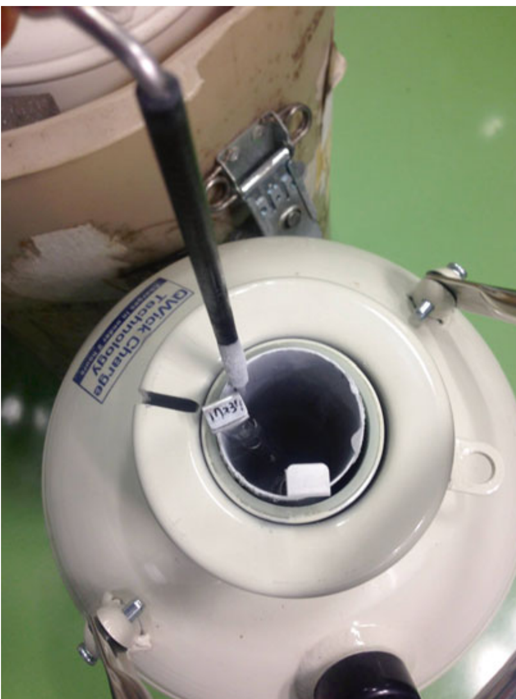

Fig. 9.4 Identify the specimen and quickly transfer to the dry shipper from the storage tank

should arise from the neck of the shipper. Plastic goblets snapped onto canes inside dry shippers will not have liquid nitrogen in them, so time in the neck of the shipper should be kept to a minimum when viewing or handling the contents (see Fig. 9.5).

Ensure the actual arrived specimens tally with the details provided by the clinic of origin. All the details regarding the received specimens should be meticulously recorded. In the event of any discrepancy noted, the clinic of origin should be informed immediately (see Fig. 9.6).

Study

A study was carried out at Rotunda – The Center for Human Reproduction to investigate difference in the viability of vitrified cryoshipped embryos and vitrified non-cryoshipped embryos in terms of survival rate and clinical pregnancy rates of frozen embryo transfers using surrogacy.

Fig. 9.5 Check for vapors emanating from the neck of the shipper on arrival

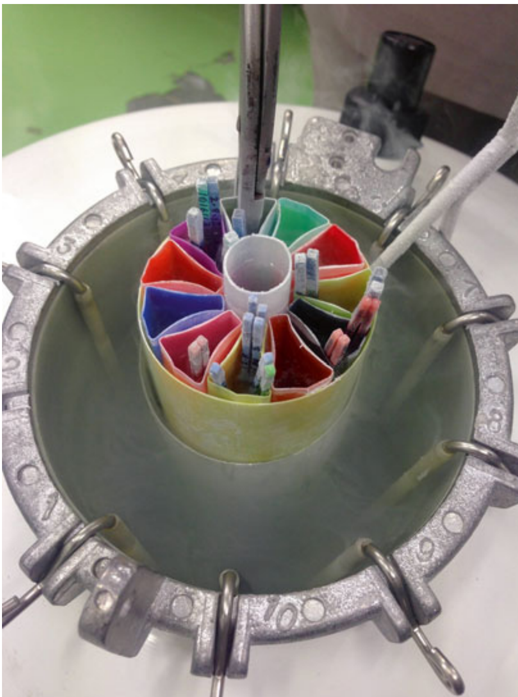

Fig. 9.6 Transfer the identified specimen to the long-term storage vessel

Table 9.1 Comparison of clinical outcomes of vitrified non-cryoshipped embryos and vitrified cryoshipped embryos

	Non-cryoshipped embryos	Cryoshipped embryos	
Total cycles	230	158	
Avg no. of embryos thawed	2.56	2.36	NS
Avg no. of embryos survived	2.48	2.22	NS
Survival rate	96.88	94.06	NS
Pregnancy rate	52.17	50.63	NS

NS nonsignificant

The embryos were cryoshipped in a dry shipper (MVE SC 4/3 V). A total of 373 vitrified cryoshipped embryos were thawed for 158 surrogacy cycles. Thawing protocols corresponding to the freezing protocols were applied, after confirming with the parent lab creating the embryos. The outcomes were compared with a parallel group of surrogacy cycles with frozen embryo transfer at Rotunda CHR, where frozen embryos had remained in-house. These embryos were cryopreserved using Cryotec method of vitrification. All the embryo transfers were done in surrogates aged 21–35 years when the optimal endometrial receptivity, as measured by transvaginal sonography, was achieved. The outcome was measured in terms of the survival rate and clinical pregnancy rate (see Table 9.1).

A total of 388 frozen embryo transfer cycles using gestational surrogacy were analyzed. 96.88 % of in-house vitrified embryos survived as compared to 94.06 % of cryoshipped vitrified embryos (p = NS). The replacement of embryos at an appropriate stage of the cycle showed pregnancy rates that were similar for the cryoshipped embryos when compared with the in-house embryos. The in-house vitrified embryos had a 52.17 % clinical pregnancy rate following transfer into gestational surrogates, as compared with a 50.63 % clinical pregnancy rate following transfer of cryoshipped vitrified embryos into gestational surrogates (p = NS).

No significant difference was found in the viability of vitrified non-cryoshipped embryos and vitrified cryoshipped embryos as the survival rate and clinical pregnancy rate were comparable in both groups. Hence, it is concluded that

Fig. 9.7 Cryoshipped vapor shipper

cryoshipping when done appropriately has no adverse effects on the viability, survival rate, and the implantation potential of the embryos (see Fig. 9.7).

Conclusion

Cryopreservation of human gametes and embryos is one of the important procedures in ART. Implementing safe handling procedures for cryopreserved reproductive cells will increase the efficiency of applied reproductive biologies. As an increasing number of IVF clinics are switching from slow freezing to the vitrification method of cryopreservation, there is a need for caution. Stress should be laid on the importance of properly handling vitrified cells as their microvolumes theoretically increase their risk for damage. Further research into the sensitivity of these vitrified cells and standardization of techniques, storage devices, and the proper handling of tissue transportation between centers is required. Emerging data continue to support that with critical care, vitrified cells can be safely transported in a dry shipper.

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10 Oocyte Banking: Current Perspectives

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Keywords
Oocyte banking • Survival • Vitrification • Fertility preservation • Cancer • Cryopreservation

Introduction

All women share a common dream of becoming a mother. It is no secret that fertility declines with age. Female fertility begins declining in the late 20s; however, conception rates remain high into the 30s. After age 35, the decline accelerates to reach nearly zero pregnancy potential by the time the woman reaches age 45. In addition, women over 35 have an increased risk of miscarriage and/or genetic abnormalities in their children as a result of age-dependent changes in egg quality.

Unfortunately, there is no medicine or technology that can "turn back the clock" and turn poor quality eggs into good quality eggs.

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Today, more and more women choose to delay childbearing for personal, medical, educational, career, or economic considerations. Based on the age-related decline in fertility, this delay can put them at risk of infertility and increases the risks in pregnancies that are conceived at an older age.

Historically, embryo cryopreservation was the only option offered to female patients. This option, while successful, has a major disadvantage about the requirement of a sperm source to create the embryos. This option is obviously closed for single women.

Fortunately, the advent of efficient and reliable oocyte freezing allows women to proactively preserve their fertility until they are ready to have children. While the decline in reproductive potential cannot be reversed, freezing of eggs at a younger reproductive age may, in effect, put the eggs in "suspended animation" until the woman is ready for conception. Oocyte banking provides women with a tool to protect their future fertility. Women can now preserve their eggs while they are still of good quality. The idea behind egg banking ("egg freezing") is that a woman can choose to have some of her eggs retrieved and then "frozen" until she is ready to use them. While there certainly can be no guarantee of a

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successful pregnancy, using cryopreserved eggs may provide a potential insurance against the biological clock and the loss of reproductive potential. Like any insurance, one may never need to use it, but it can provide some degree of peace of mind knowing it is there if needed.

Considerations for Oocyte Banking

Donor Oocyte Programs

Oocyte donation is one of the last resorts in IVF treatment for couples challenged with infertility problems. It is an efficient alternative to using own oocytes in IVF treatment for different indications. Unfortunately, "traditional" (fresh) egg donations are challenged with inefficiency, difficulties of synchronization, very long waiting periods, and lack of quarantine measures. Because of the development of an efficient oocyte cryopreservation technique, it is now possible to cryo-store donor eggs, maintaining their viability and allowing their use whenever there is demand. Donor egg cryobank has several benefits including the ease of use for donors and recipients, higher efficiency, being more economical, and avoidance of the problem of synchronization. All these features make it likely that this approach becomes the future standard of care.

Fertility Preservation for Medical Reasons

Oocyte banking may be offered to women of reproductive age who are scheduled to undergo medical treatment that could lead to premature decline of ovarian function [1, 2]. Due to significant improvements in cancer treatments, patients affected by oncologic disease are living longer, fuller lives. As a result, the fertility potential of reproductive-age women affected by cancer has become an increasing focus for those who counsel and treat such patients. Also women with a family history of early menopause can consider egg banking. Moreover, it may also offer alternatives for infertile patients who are subject to ovarian hyperstimulation syndrome or premature ovarian failure. Young women with a low ovarian reserve, ovarian surgery, and endometriosis can avail to oocyte freezing [3]. It can also be used in cases of unavailability of a male gamete on the day of ovum pickup.

Fertility Preservation for Social and Ethical Reasons

Oocyte cryopreservation solves the legal and ethical problems associated with the creation and storage of embryos in patients undergoing in vitro fertilization procedures. It is also beneficial for women who want or need to delay childbearing in order to pursue educational, career, or other personal goals. Because a sperm source is not needed before oocyte cryopreservation, women without a male partner may consider this option.

Cryopreservation of Oocytes

The potential advantages of being able to cryopreserve oocytes have been apparent for many decades. When first introduced in the 1980s, the ability of a cryopreserved oocyte to be fertilized and result in a live birth was compromised by poor oocyte survival and poor fertilization rates [4–6]. Dr. Christopher Chen of Australia reported the world's first pregnancy in 1986 using previously frozen oocytes [7]. Oocytes are susceptible to cryodamage, which collectively entails cellular damage caused by mechanical, chemical, or thermal forces during the freezing and warming process. The metaphase II (M-II) oocytes are fragile owing to its large size, water content, and chromosomal arrangement. Technical difficulties associated with the unique properties of the mammalian oocyte initially retarded rapid development in this area, but recent advances have overcome many of the problems.

Two methods, slow freezing and vitrification, have been used successfully for oocyte cryopreservation. Slow freezing traditionally has been the most commonly used method to cryopreserve oocytes. Ultrarapid freezing or vitrification represents a potential alternative freezing method. A stage has now been reached where oocyte

cryopreservation can be considered an important component of human-assisted reproductive technology.

Oocyte Banking

Recent advances in oocyte cryopreservation have allowed more women to pursue fertility preservation. Improvements in cryopreservation techniques have resulted in significantly improved outcomes in patients opting for oocyte banking [8–11]. Currently, more than 50 % of IVF centers in the USA offer oocyte cryopreservation for cancer patients [10].

In the fall of 2009, the American Society for Reproductive Medicine (ASRM) issued an opinion on oocyte cryopreservation concluding that science holds “great promise for applications in oocyte donation and fertility preservation.” The ASRM noted that from the limited research performed to date, there does not appear to be an increase in chromosomal abnormalities, birth defects, or developmental deficits in the children born from cryopreserved oocytes. The ASRM recommended that, pending further research, oocyte cryopreservation should be considered an experimental procedure [12]. However, the ASRM did support the use of oocyte cryopreservation as a “fertility preservation strategy for women with cancer and other illnesses requiring treatments that pose a serious threat to their future fertility” [13]. In October 2012, the ASRM lifted the experimental label from the technology, citing success rates in live births, among other findings [14].

Procedure for Oocyte Banking

Oocyte Retrieval

The egg retrieval process for oocyte cryopreservation is the same as that for in vitro fertilization. This includes a few days of hormone injections that stimulate ovaries to ripen multiple eggs. Thereafter, the eggs are removed from the ovary using an ultrasound-guided needle through the vagina under sedation.

Oocyte Freezing

Following oocyte retrieval, oocytes are prepared for cryopreservation. Two methods of oocyte cryopreservation are currently in use, slow freezing and vitrification. With the slow-freezing method, the oocyte is placed in a low concentration of cryoprotective solution, and the oocyte is then slowly frozen in a programmable freezer. In vitrification, the oocyte is placed in a high concentration of cryoprotective agents and then rapidly cooled using liquid nitrogen, resulting in a solid glass-like cell, free of ice crystals. This vitrified state has resulted in improved oocyte survival and pregnancy rates from frozen oocytes in IVF. The thawing process is also ultrarapid in order to avoid ice nucleation.

Timing

The duration of treatment, from the start of stimulation to oocyte retrieval, is approximately 14 days. In case of cancer patients, chemotherapy can be started after 1–2 days of oocyte retrieval [15].

Cost

In addition to the IVF cycle fees, the initial cost of freezing and storage may add to the total charge, and there are additional fees at the time of thawing and transfer. Costs vary from center to center. As with embryo cryopreservation, insurance coverage is widely variable.

Success Rates

Success Rates with Slow Freeze Compared with Vitrification

Current evidence suggests that vitrification may result in higher survival, fertilization, implantation, and pregnancy rates than slow freezing [16, 17]. Therefore, the vitrification technique is most often utilized for oocyte cryopreservation. However, some clinics report equivalent success with slow freeze and vitrification in observational studies [8], and it is likely that clinic-specific

success rates may vary with different methods of cryopreservation.

Dr. Kuwayama has introduced several novel procedures to vitrify oocytes and embryos, using minimal volume approach, including the Cryotop method and the Cryotip method. High post-thaw survival and pregnancy rates by the Cryotop method have been repeated at many IVF faculties throughout the world [18–20]. With major advances, the new optimized method "the Cryotec method" has been developed for oocyte and all the stage of pre-implantation embryos. This open-style method is recognized as easy, simple, and repeatable for all. These improvements result in very high functional survival of oocytes, assuring high rates of fertilization after intra cytoplasmic sperm injection (ICSI) and high rates of pregnancy after embryo transfer [11].

Success of IVF with Cryopreserved Oocytes Compared with Fresh Oocytes

The results of egg donation using slow freezing are encouraging; outcomes are not yet comparable to a fresh egg donation treatment. Vitrification on the other hand appears to provide high survival rates and comparable fertilization, embryo development, and implantation to fresh oocytes [9, 21–23].

The Impact of Maternal Age on Oocyte Cryopreservation Success

Success of an oocyte cryopreservation cycle is highly dependent upon the patients' age and baseline fertility evaluation. As with fresh oocytes, there is an expected decline in success with cryopreserved oocytes of increased age [24, 25]. There are no comparative trials assessing success with cryopreserved vs. fresh oocytes by age.

Risks

Medical risks are similar to that for embryo cryopreservation. There is a small possibility that during egg retrieval no eggs are collected or the collected eggs are not mature and hence not suitable for freezing. In addition, there is a risk that the oocytes may not survive thawing, not fertilize, or not result in a pregnancy in the future. The risks associated with ovarian stimulation and oocyte retrieval also apply. The risks of ovarian hyperstimulation syndrome (OHSS) are very low, as embryo transfer is not being performed in most individuals cryopreserving oocytes [26].

An estimated 3.5 million children have been born to date using assisted reproduction technologies. While there are a limited number of established pregnancies and deliveries derived from cryopreserved oocytes, perinatal outcome data are reassuring [27]. Recent studies have shown that the rate of birth defects and chromosomal defects when using cryopreserved oocytes is consistent with that of natural conception [28, 29]. While short-term data appear reassuring, long-term data on developmental outcomes and safety data in diverse (older) populations are lacking.

There also are theoretic infectious disease concerns with the use of open vitrification methods. However, to date infectious transmission has not been observed in reproductive tissues from this technique [30].

A miscarriage can also occur after pregnancy is achieved similar to a natural conception. It is known that all medical procedure and treatments carry some element of risk.

Benefits of Donor Oocyte Banks

1. A large donor pool.
2. Recipients are guaranteed 5 to 7 mature eggs per cycle.
3. Low risk of cycle cancellation (<3 %).
4. Oocytes in "quarantine" for 6 months until confirmation of serology of the donor.

Ethical Issues

In case of donor oocyte banks, the argument against commercialization of human gametes is that it is morally repugnant to put a price on that which creates life. Also, the argument against anonymous gamete donation is that it is not in the best interest of the children conceived from the use of these donor gametes who have the right to know their biological origins. Many other issues

remain unanswered, including responses to possible side effects on the egg donors and support for children of donated eggs who may grow up with questions about their biological origins.

Also, marketing this technology for the sole purpose of circumventing reproductive aging in healthy women may give women false hope and encourage women to delay childbearing. Patients who wish to choose this technology should be carefully counseled.

Legal Issues

Laws regarding donor oocyte banking vary from country to country. In some countries, oocyte donation is legal, whereas in others it is not. Desperation has led some to reportedly pay up to $100,000 for eggs overseas in countries where payment for donation is legal. It has raised concerns about vulnerable people being exploited in donor arrangements, with little oversight of the procedure or legal protection for women and their donor-conceived children.

Canada – In 2004 the Assisted Human Reproduction Act became law and prohibited the purchase of donor gametes from a donor or a person acting on behalf of a donor; most donor sperm used in Canada has been imported via the USA or other countries.

Japan – In 2003, a health ministry panel endorsed egg donations by anonymous third-party donors on the condition that relevant laws are developed. But in the absence of such laws, there is no legal framework for defining parental relations or providing support for children of donated eggs.

Italy – Laws prohibit embryo banking; thawed oocytes can be successful and safe in helping patients achieve live birth.

USA – Anonymous egg donation is legal.

Duration of Storage

There is not much data available regarding the effect of duration of storage on oocyte cryopreservation survival and pregnancy. A study by Parmegiani et al. assessed oocyte cryopreservation efficacy with duration of storage. In this study, no difference was observed in survival, fertilization, cleavage, embryo quality, implantation, and live-birth rates in oocytes cryopreserved with slow freeze and thawed after up to 48 months compared to earlier dates of thawing [31].

Conclusion

The success of oocyte cryopreservation has improved dramatically over the past decade, and preliminary data for safety are reassuring. Egg banking holds promise for women who want to preserve their fertility and delay motherhood for various social reasons such as education and career development and for medical reasons usually ahead of cancer treatment. Egg banking will improve chances of conception with one's own eggs at a more advanced reproductive age. It should be noted that the age of the woman at the time of egg freezing is a very important factor. Success rates with oocyte cryopreservation appear to decline with maternal age consistent with the clinical experience with fresh oocytes.

For patients who are facing infertility due to medical reasons, oocyte cryopreservation may be one of the few options available and therefore is recommended under these circumstances with appropriate counseling. However, there are not yet sufficient data to recommend oocyte banking for the purpose of deferring childbearing in healthy women because there are no data to support the safety, efficacy, ethics, emotional risks, and cost-effectiveness of oocyte cryopreservation for this indication.

Oocyte cryopreservation has become a mainstream fertility technique with excellent results, especially in oocyte donation programs. Egg donation has high and comparable pregnancy and delivery rates when using fresh and vitrified oocytes. In an egg donation program, oocyte banking has many advantages. Vitrification of donor oocytes is the solution for the logistic problems commonly occurring in an egg donation program. Oocyte banking is a promising new phenomenon. The benefits of a donor egg bank make it likely that this approach becomes the future standard of care.

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11 Vitrification of Human Embryonic Stem Cells

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Keywords
Vitrification • Human cells • Human embryonic stem cells • Stem cells • Cryopreservation

Introduction

Pluripotent human embryonic stem cells (hESCs) have an unlimited capacity for self-renewal and, in culture, to maintain their pluripotent capacity to differentiate into cell types from all three germ layers [1]. Though first isolated from surplus in vitro fertilized blastocysts in 1994 [2], it was not until 1998 that the first stable hESCs cell line was established by Thomson et al. [3]. The capacity of these cells to undergo virtually infinite expansion and asymmetric cell division with differentiation into cells of ectodermal, endodermal, and mesodermal origin was confirmed 2 years later [4]. Since then, hundreds of stem cell lines have been derived worldwide from blastocysts of fresh and cryopreserved supernumerary embryos as well as from morula [5], single blastomeres [6], arrested embryos, and embryos discarded after preimplantation genetic diagnosis [7, 8]. PGD-affected embryos are not used for transfer or cryopreservation, but they can be a valuable source for the derivation of hESCs lines [9].

Human ESCs hold tremendous promise as not only a tool for understanding diseases but also as a basis for cell-based therapies [10]. The ability of stem cells from any source, but more particularly the embryonic stem cells, to produce a theoretically unlimited supply of normal differentiated cells has focused attention on the potential importance of these cells in both toxicology and drug discovery [11, 12], as well as tissue engineering [13, 14] and gene and cellular therapy [15, 16] for a wide range of human diseases including Parkinson's and other neurodegenerative diseases [17–19] and diabetes [20, 21] and cardiac and vascular therapy [22, 23]. In the USA the Food and Drug Administration approved in 2009 the first US clinical trial of hESC-based therapies in humans for the treatment of spinal cord injuries [24].

In early studies, using a standard slow cooling protocol, Reubinoff et al. [25] reported 16 % recovery after freezing and thawing (as measured by the number of colonies recovered 2 weeks after thawing). Zhou et al. [26] also reported similar results with only slightly higher recovery

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(approximately 23 %, based on the number of colonies recovered at day 9, post-thaw).

Human stem cells and mesenchymal stem cell lines are routinely subcultured and have been successfully cryopreserved as an undifferentiated, single-cell suspension [27]. In contrast, hESCs have not been amenable to passaging or cryopreservation as single-cell suspensions. As a result of this limitation and the poor results obtained through conventional cryopreservation, vitrification has been adopted as the method of choice for the preservation of hESC lines [1].

Cryopreservation by Vitrification

The adoption of this vitrification method as the preferred cryopreservation method for hESCs is largely due to comparative studies developed by three groups, two of which indicated rates of recovery of more than 75 % undifferentiated colonies for vitrified hESCs compared to around 5 % after slow cooling [25–27] and due to the undisputed success of vitrification in cryopreservation of oocyte and embryo worldwide. The vitrification protocols reported in these studies were very similar, all derived from the Kuwayama's work developed for bovine ova and embryos [28] and modified by Reubinoff et al. [25] for application to hESCs. This same basic preservation methodology has been applied to hESCs by a variety of investigators [27, 29].

In essence, this protocol requires the stepwise exposure of hESCs colony fragments to two vitrification solutions of increasing cryoprotectant concentration, the common components of which are DMSO and EG. Briefly, colonies of hESCs (100–400 cells) are placed in a vitrification solution composed of 20 % DMSO + 20 % EG + 0.5 mol/l sucrose after equilibration with a solution containing lower concentration of DMSO + EG. The colonies are loaded into straws and plunged into liquid nitrogen. Post-thaw recoveries using this method have demonstrated higher post-thaw rates (94 % colony attachment) when compared to conventional cryopreservation methods (6 % colony attachment) [29].

This approach involves several problems: (1) preservation in straws is open/nonsterile; (2) the process is very labor intensive as colonies have to be physically moved from one solution to the next during introduction and removal of the vitrification solution; and (3) traditional formulations use animal proteins in the solution.

Richards et al. [27] developed a closed straw method and used human serum albumin (vs. fetal bovine serum) to obtain post-thaw recovery of colonies comparable to that obtained using conventional vitrification techniques and have improved the overall technique.

Similar results were observed by Zhang et al. [30] when a solution containing 10 % DMSO + 90 % knockout serum replacement was supplemented by 0.2 mol/l trehalose. Adding trehalose to the solution increased the recovery of colonies from 15 to 48 %. Several methods were described [29, 31–34], including surface-based approaches [35].

Vitrification and Scale-Up

New technology to facilitate high-efficiency processing of cells for preservation will be needed. Protocols must be developed to permit effective methods of cell preservation in different contexts.

The limitations on sample volume, necessary to achieve the required cooling rates, and the need for vitrifying the embryonic stem cells as small clumps of stem cells at best permit the production of only laboratory-scale cell banks by the OPS method. This process is time-consuming, operator dependent, and, because of the short exposure times to cryoprotectant, prone to both failure and inconsistency in the final product [1]. Attempts have been made to address this, and some success has been achieved on the scale of production. Heng et al. [36] proposed a design for a culture plate with detachable wells in which whole adherent colonies could be vitrified.

Li et al. [37] described a bulk vitrification method with cell strainer that could cryopreserve 136 ± 23.4 cell clumps at one time (round), which was 30 times as high as those for OPS method (4 ± 1.5). After thawing, bulk-vitrified human

embryonic stem cells exhibited high survival rate up to 94.3 %, comparable with the OPS method, and exhibited no difference in the rate of reattachment or in the degree of differentiation (measured at day 7) or in the pluripotency of the surviving cells.

A refinement of this technique by the same author, using customized cryovials fitted with stainless steel mesh, produced similar results [34]. Both methods allow an increase in the quantity of hESCs preserved at any one time, bringing us closer to a commercial scale-up.

Conclusions

The recent start of some long-awaited clinical trials using hESC-derived cells in both the UK and USA highlights the potential use of these cells as therapeutic agents. These human stem cells would probably be in a near future the key to treat diseases with limited treatment options presently. As the clinical need for stem cells to treat human disease continues to grow, the need for effective and clinically relevant methods for preserving those cell types will also continue to grow. Furthermore, even as the methods by which to produce these cells in the scale required continue to be developed, their use in drug screening and toxicology assays is also set to concomitantly increase [38].

Cryopreservation is very important but just a small part of the process of producing and maintaining stem cells and their derivatives for therapy. While the process is routine for hematopoietic stem cells and largely worked out for mesenchymal stem cells, at least for autologous use, cryopreservation of hESCs is only now really being explored.

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