

Overview of Cryobiology in ART

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Abstract

Cryopreservation allows the transfer of a limited number of embryos back to the uterus and the storage of the remaining embryos for future use, thus maximizing the cumulative effectiveness of an *in vitro* fertilization (IVF) cycle. In addition, cryopreservation makes feasible the postponement of embryo transfer (ET) in a future cycle, thus decreasing the incidence of ovarian hyperstimulation syndrome in high-risk patients, while it maintains the probability of pregnancy. The main problem during embryo cryopreservation is the formation of intracellular ice, which can lead to cell damage and developmental arrest. To overcome this problem, different cryopreservation protocols, such as slow freezing or vitrification, along with different types of cryoprotective solutions, such as propanediol and dimethyl sulfoxide (DMSO), have been developed. In this way the cells are protected against potential injuries occurring at subzero temperatures.

Keywords: Cryopreservation, cryoprotectant, cryoinjury.

INTRODUCTION

Over past two decades freezing techniques in human *in vitro* fertilization (IVF) have improved due to increased knowledge about biochemistry and membrane structure of germ cells. However, still the pregnancy outcome in humans is quite low as compared to rodents and cattle. During cryopreservation, cells are exposed to numerous stresses including mechanical, thermal and chemical^{1,2} which can lead to compromised cell function and cell death. So far, cryopreservation of embryos seems to have no negative impact on perinatal outcome and early infant development compared with fresh embryo transfer. The available data do not indicate an elevated congenital malformation rates, but it remains unclear if embryo freezing poses long-term risks to children conceived so far. Cryopreservation provides a continuous source of tissues and genetically stable living cells for a variety of purposes, including research and biomedical processes. In Assisted Reproductive Technology, cryopreservation techniques are being used for freezing of sperms, eggs, embryos, blastocyst and ovarian and testicular tissue.

In 1866, an Italian military physician Mantegazza documented the observation that human spermatozoa become immotile when cooled in snow.³ He subsequently proposed that it might be possible for a soldier to father a child, even after his death, by cooling and storing spermatozoa. A major breakthrough occurred with the discovery that glycerol imparts cryoprotective properties to spermatozoa during freezing and thawing.⁴

Successful cryopreservation of mouse embryos was reported in 1972 by Whittingham et al.⁵ They used 1.5 M dimethyl-

sulfoxide (DMSO) as the cryoprotective agent combined with slow cooling rate ($-0.3^{\circ}\text{C}/\text{min}$ to -80°C) and stored embryos in liquid nitrogen. In 1983, Trounson and Mohr reported pregnancy following freezing and thawing of 8 cell embryo in humans.⁶

BASIC DEFINITIONS

Cryobiology is a branch of biology that studies life at below-normal temperatures. Usually cryobiology is considered to deal with the effects of freezing and thawing. However, any temperature below what is normal for any given living system falls into the realm of cryobiology, including fields such as hypothermia, hibernation, natural frost hardiness of insects and plants, and medical organ preservation in ice.

Cryopreservation is the process of preserving and storing the living systems in a viable condition at low temperatures for future use. Traditionally, cryopreservation has meant preservation by freezing, and the word is still used with this meaning in many cases. However, the term can also cover preservation by vitrification, or ice-free cryopreservation.

Cryogenics is a branch of physics which studies the causes and effects of extremely low temperatures. It relates to cryotechnologies such as producing liquefied gases as well as to many other low temperature physical effects such as superfluidity and the behavior of Bose-Einstein condensates.

Cryonics is the practice of keeping a clinically dead human body or brain at an extremely low temperature in the hope of later restoring it to life with the help of future medical technologies.

Vitrification is preservation at extremely low temperatures without freezing. Freezing involves ice crystal formation, which damages delicate structures such as blood vessels. Vitrification instead involves the formation of a glassy or amorphous solid state which, unlike freezing, is not intrinsically damaging even to the most complicated of living systems.

THERMODYNAMICS

Temperature is a measurement of the internal energy in a physical system. It is this internal energy in fluid systems that allow molecules in fluids to tumble, twist, disassociate from one another, move from place to place in the fluid, and chemically react with other molecules. As the temperature is reduced, less and less energy exists in the system to drive these kinds of molecular motions. In many systems such as pure water, temperature reduction below a certain point results in an abrupt reorganization of the fluid medium into an organized solid lattice known as a crystal. This is known as freezing. In other systems, this does not happen. Instead, temperature reduction just causes more and more slowing of molecular motions, less and less molecular mobility, and slower and slower chemical reaction rates until a critical temperature is reached below which there is insufficient energy for the most mobile molecules in the fluid to move appreciably over the time scale of a typical laboratory observation. At this temperature, the “glass transition temperature,” the system almost completely loses its fluidity and becomes a “solid liquid,” which is more formally known as a “glass,” and is said to have “vitrified.” A glass is like a snapshot of the liquid state, and is essentially a liquid in which molecular rearrangements are practically arrested. At several degrees below the commonly observed glass transition temperature, molecular motions are so slow that changes are nil for all practical purposes even over time spans of several hundred years or more.

PHYSICS

1. Water moves from a region of low osmolarity to a region of high osmolarity.
2. Nucleation temperature is the temperature at which water forms ice crystals.
3. Solutes dissolved in the solution decrease the nucleation temperature of the solution. Such solutions tend to super cool and form ice rapidly at very low subzero temperatures.

CONCEPT OF LATENT HEAT

Latent (Hidden)

The three physical states of matter that we normally encounter are solid, liquid, and gas. Water can exist in all three physical states at ordinary temperatures on the Earth’s surface.

When water is in the vapor state, as a gas, the water molecules are not bonded to each other. They float around as single molecules.

When water is in the liquid state, some of its molecules bond to each other with hydrogen bonds. The bonds break and reform continually. When cooled water converts to the solid state (ice), the molecules are bonded to each other in a solid crystalline structure. This structure is six-sided, with each molecule of water connected to four others with hydrogen bonds. Because of the way the crystal is arranged, there is actually more empty space between the molecules than there is in liquid water, so ice is less dense. That is why ice floats.

Each time water changes physical state, energy transfer is involved. In the vapor state, the water molecules are very energetic. The molecules are not bonded with each other, and move around as single molecule. In the liquid state, the individual molecules have less energy, and some bonds form, break, then reform. At the surface of liquid water, molecules are continually moving back and forth from the liquid state to the vapor state. At a given temperature, there will be equilibrium between the number of molecules leaving the liquid, and the number of molecules returning.

In solid water (ice) the molecules are locked together in a crystal structure: a framework. They are not moving around, and contain less energy.

When water converts into ice which has more orderly arrangement and minimal movement of the water molecules there is release of energy. This energy is called latent heat.

Similar change occurs when ice converts back to water. Here energy is absorbed which will break hydrogen bonds which are holding all the water molecules together in ice crystals.

Similarly, to evaporate water we put energy into it. The individual molecules in the water absorb that energy, and get so energetic that they break the hydrogen bonds connecting them to other water molecules.

To summarize we have to remember that each time water changes state, energy is absorbed or released. This energy is latent heat. Latent heat is the energy absorbed or released when a substance changes its physical state. Latent heat is absorbed upon evaporation, and released upon condensation to liquid (as in clouds). Latent heat is also absorbed when water melts, and released when it freezes.

While converting solid water (ice) to liquid water at 0°C, it will require about 80 calories of heat to melt one gram of ice, and the 80 calories will be released when the liquid water is frozen to the solid state. This energy is hidden latent heat. This plays an important role during nucleation and super cooling of the freezing solutions and recrystallization during thawing.

BIOLOGY OF CRYOFREEZING

Cryobiology succeeds in preserving living systems at cryogenic temperatures when the destructive effects of low temperatures can be limited sufficiently during cooling. The challenge of cryopreservation, in other words, is to pass through a specific temperature range of vulnerability to low-temperature and reach a temperature sufficiently low to prevent any further change over virtually unlimited periods of time.

The cryopreservation of gametes and embryos involves an initial exposure to cryoprotectants, cooling to subzero temperatures, storage, thawing, and finally, dilution and removal of the cryoprotectants, with a return to a physiological environment which allows their further development.

The increasing demand for successful cryopreservation methods results from the lower number of embryos being transferred in order to reduce multiple pregnancy rates in ART. A successful freezing and thawing procedure ensures the maintenance of the cell's structural integrity as well as its functional characteristics. Proper management of the osmotic pressure is crucial for success and it is important to avoid damage from intracellular ice formation. If the dehydration process has been inadequate, large, intracellular ice crystals are formed. Therefore the cells are dehydrated before and during the cooling procedure.⁹

We have to always remember 3 basic facts while cryofreezing human gametes and embryos.

1. Cell membrane is biologically permeable to liquid water but not to ice.
2. Different cells due to varying cell membrane constituents and cytoplasmic osmolarities have different permeability to water.
3. Embryos have a low permeability to water and a low ratio of surface area to volume as compared to other body cells. An important factor that influences the response of a cell to freezing is the ratio of its surface area to volume of the cell. A human oocyte with a diameter of 120 μm has a volume of $9.05 \times 10^5 \mu\text{m}^3$ and a surface area of $4.5 \times 10^4 \mu\text{m}^2$ with surface area to volume ratio of only 0.05. This requires longer freezing cycle and more chance of injury to the cell.

CRYOPROTECTANTS

All the freezing methods developed today rely on the presence of one or more cryoprotectants in buffered carrier medium. 1, 2-propanediol, glycerol, DMSO (dimethyl sulfoxide) and ethylene glycol are intracellular (or penetrating) cryoprotectants. Their molecular weight is relatively low ($\text{MW} < 100$). Extracellular cryoprotectants are compounds that, as a result of their size or polarity, remain in the extracellular solution. These include large molecules such as sucrose, proteins, and lipoproteins. The mode of action of cryoprotectants is complex. They bind to water and reduce the toxic effects of high concentrations of other compounds. Furthermore they protect cells during slow cooling when the cells are very dehydrated and are surrounded by concentrated salts. At high concentrations, cryoprotectants minimize the damage caused by ice formation, as they cause the water to form a glass structure rather than ice crystals. Mechanism of action of cryoprotectants is as enumerated below.

Permeating Agents

Glycerol, 1-2 Propanediol (PROH), DMSO, Ethylene Glycol (EG).

Mechanism of Action

1. Reduction in electrolyte concentration.
2. Lowering of temperature of ice nucleation.

Nonpermeating Agents

Sucrose, Proteins

Mechanism of Action

1. Osmotic dehydration during cooling.
2. Restrict water influx by acting as an osmotic counter force.

PRINCIPLES OF CRYOBIOLOGY

A basic principle of cryobiology is that the extent of freezing damage depends of the amount of the free water in the system and the ability of that water to crystallize during freezing. During freezing, most of the water changes to ice, and cellular metabolism ceases. Cryopreservation can be done using conventional slow freezing techniques or by newer techniques as vitrification.

Conventional slow cooling: It is based on the principle of dehydration, where cooling rates are optimized to remove water from the embryo, preventing cryoinjury from ice crystal formation while minimizing chemical toxicity, and osmotic stress from exposure to high concentration of salts. In the process of conventional cryopreservation after initially equilibration in cryoprotectant (CPA) for 5 to 10 minutes at 20 to 25°C, seeding is done at -5 to -9°C followed by further cooling at 0.3 to 0.6°C/min down to -33 to -40°C, followed by plunging into liquid nitrogen.

Vitrification: It is now regarded as a potential alternative to the conventional slow freezing method. It is a process of rapid cooling of a liquid by short exposure with a high concentration of CPA to achieve a glass like solid state. It has the advantage of preventing ice crystal formation and this rapid freezing technique also minimizes chilling injury and osmotic shock to the embryo. Two conditions usually required to allow vitrification are an increase in the viscosity and a depression of the freezing temperature. These conditions are achieved by addition of CPA which acts like antifreeze. Vitrification usually requires the addition of CPA prior to cooling. Other components required for vitrification are basal medium buffered with phosphate or HEPES, macromolecules, sugars and proteins. Vitrification was first used for preserving embryos from the mouse in 1985.⁸ Since then the techniques has undergone various improvements by using less toxic and more permeable chemicals, by using combination of CPA to reduce toxicity, using a stepwise approach to equilibration and increasing cooling and warming rates.⁹

Ethylene glycol has been widely used as a CPA in both slow freezing and vitrification methods due to its high permeation ability and low toxicity compared with other CPAs.^{10, 11}

Table 1: Comparison of embryo vitrification with conventional slow cooling

Factors	Vitrification	Conventional slow cooling
1 Time consumed	Less (10 minutes)	More (3 hours)
2 Instruments	Inexpensive	Expensive
3 Sample volumes	1 to 2 μ l	100 to 250 ml
4 Cooling rates	15,000 to 30,000 $^{\circ}$ C/min	0.3 to 0.6 $^{\circ}$ C/min
5 CPA concentration	High	Low
6 Ice crystallization	No	Yes
7 Direct contact with LN2	Depends upon the carrier being used	No
8 Mechanical damage	Less or none	More
9 Chemical damage	More	Less

Martinez et al recorded better pregnancy rates using ethylene glycol (1.5M) with sucrose (0.1M), as compared with sucrose (0.3M).¹² Better embryo survival rates have been reported by use of vitrified bovine embryos than by conventional slow freezing.¹³ Agca and Monson et al reported that pregnancy rate of conventionally frozen embryos was lower than that of fresh or vitrified embryos.¹⁴ The table shows comparison of embryo vitrification with conventional slow cooling (Table 1).

STEPS OF CRYOPRESERVATION

Step 1

Exposure and equilibration with permeating and nonpermeating cryoprotectants in increasing concentrations.

Their cryoprotective activity seems to be based on their ability to decrease the freezing point of solutions and to reduce the amount of salts and other solutes present in the remaining liquid phase, below the freezing point of the solution (beginning of ice formation) and above the eutectic points of the salts. Thus the incidence of lipoprotein denaturation is reduced. Cryoprotectants may also decrease the velocity of crystal formation and modify their shape into a smoother pattern. They may also act by a direct association with cell membranes. Sucrose is often used together with other cryoprotectants. It is not able to penetrate through the cellular membrane and its presence in extracellular media can exert a significant osmotic effect.

Step 2

Cooling to subzero temperature and inducing orderly ice formation following nucleation. This avoids supercooling of the solution.

When the temperature is lowered following seeding, the composition of the nonfrozen fraction of the solution changes because only pure water molecules crystallize on the growing ice crystal front. Salts and other solutes are not incorporated into the ice and as a result the osmolarity of the nonfrozen fraction increases. The amount of water which remains unfrozen decreases as the temperature falls. As water is lost from the nonfrozen fraction, embryos and oocytes remain in osmotic equilibrium with their external environment by losing water.

The cells become dehydrated. By adjusting the cooling rate and duration of cooling it is possible to control how much water is lost by the cell. The aim is to reduce intracellular water, to a level which is compatible with cell survival after storage in liquid nitrogen.

The optimal cooling rates for cells differ as a result of (1) the composition and permeability characteristics of the cell membrane; (2) the surface to volume ratio of cells (3) the difference in osmotic pressure between the two sides of the membranes. These factors should ideally be taken into account in dehydrating and rehydrating cells.

Cryoprotective solutions usually reach temperatures as low as -15° C before ice is formed. This is called supercooling. Avoiding supercooling is essential for success of the procedure. A biological freezer then lowers the temperature around these containers (vials/straws) at a predetermined controlled rate. When slow cooling is used for oocytes or embryos the solution is usually "seeded" manually to induce ice formation at temperatures between -5° C and -7° C. This is most commonly achieved by touching the wall of the straw or vial with a cold object, e.g. forceps in liquid nitrogen. This process is called seeding or ice nucleation. The aim is to create the first ice crystal(s) at a site at some distance away from the embryo. This prevents the whole solution from supercooling. Once ice formation has been initiated, the ice propagates itself from this point throughout the rest of the solution. Manual seeding is preferable to spontaneous ice formation because it prevents deep supercooling and starts the dehydration process, both of which, reduce the likelihood that ice form inside the cell(s). It also allows the latent heat of crystallization to be released gradually.

In solutions which are seeded at a high subzero temperature large ice crystals spread slowly through the solution. Under these conditions, there is little risk of the ice spreading into the intracellular compartment because cells have a marginally higher solute concentration, and therefore lower freezing point, than the solution. By contrast when ice formation starts in a deeply supercooled solution the ice propagates very rapidly. The temperature of the solution rapidly rises as the latent heat of crystallization is released throughout the solution. The ice crystals, which form, tend to be small in size and may form in both the solution and in cells. Seeding is highly dependent upon the thickness of the straw (thin, thick), and vial. To get the optimal ice crystals that do not pierce the oocytes, embryos or blastocysts, following guidelines are advocated.

- Thin straw– Seed 1 to 2 minutes after reaching the seeding temperature, hold for total of 10 minutes.
- Thick straw– Seed after 7 minutes, hold for 3 minutes.
- Vial, seed after 10 minutes and hold for additional 10 minutes.

Step 3

Holding the cells at a specific temperature (soakage period) allowing equilibration of the intracellular contents with cryoprotectants.

Holding oocytes or embryos at the seeding temperature, is to allow the solution to stabilize at the correct temperature and to prevent disorderly and harmful ice-crystal formation when we seed the solutions containing germ cells and embryos. This helps us to attain higher survival rates.

Step 4

Storage of the Specimen at –196°C

Liquid nitrogen (–196°C) holds specimens at a temperature at which there is virtually no movement of atoms or molecules. It has been speculated that at these temperatures only radiation (e.g., background cosmic radiation) is likely to cause modifications to stored tissues. At temperatures above approximately –130°C, atoms and molecules are able to move. Temperatures of –90°C and above allow ice crystal growth and even short periods of exposure to such “high” temperatures can cause lethal damage to embryos.

Step 5

Thawing of the specimen at the room temperature or /and dipping the freezing container (cryovials, freezing straws) in water at 37°C.

During thawing, ice crystals change before melting, water molecules run from small crystals to the surface of big ones, which grow in dimensions and roughness and may destroy the cells. Thawing has therefore to be very rapid, to limit the period during which the changes of the water phase may occur.

During the freezing protocol when the fall in temperature is low (0.3°C/min) until –80°C, a high degree of cell dehydration is obtained and thawing must be slow as well (4 to 8°C/min) to allow a progressive rehydration of the cells. Therefore, the whole procedure takes a long time (~6 hours). If rapid thawing is attempted cell lysis can occur due to rapid rehydration and osmotic stress.

Leading cryobiological schools recommend limiting the slow fall in temperature during freezing in programmable planner till temperature of (–30°C to –40°C) is attained and then plunging the embryos directly in liquid nitrogen. Small internal ice crystals do form, but they are limited, as a partial cell dehydration has already occurred during the initial cooling. Cell dehydration also has been optimized by the addition of sucrose at room temperature before freezing. Thawing will have to be very rapid (300 to 400°C/min) to avoid any recrystallization.

These high thawing speeds are obtained in practice by just holding the ampoules or straws, containing the embryos at room temperature for several seconds or by directly exposing them to 37°C in a water bath.

Step 6

Serial dilution and removal of cryoprotectants using cryoprotectants in reducing concentrations.

Cryoprotectants have to be removed, usually by a stepwise procedure, lowering progressively their concentrations and allowing them to leave the cells without excessive rehydration or osmotic stress. When a cell, which contains cryoprotectants, is placed in medium containing a lower concentration of cryoprotectants, water enters the cells to dilute the cryoprotectants more rapidly than the cryoprotectants can leave the cells. This cause cells to swell or even burst. The cryoprotectants are removed by placing the cells in a high concentration of a nonpermeating molecule such as sucrose. The high extracellular concentration of the nonpermeating molecule counter-balance the high concentration of cryoprotectant in the cell, as it reduces the difference in osmolarity between the inside and the outside of the cell. The use of high concentrations of sucrose usually allows the cryoprotectant to be removed in one step, thus it is both simpler and faster than those procedures, which remove the cryoprotectant by dilution alone.

EVENTS DURING FREEZING

1. Extracellular ice formation occurs following nucleation thus increasing the osmolarity in the surrounding medium.
2. Water moves out of the cell to surrounding media which now has high osmolarity as compared to surrounding medium.
3. Intracellular ice formation is thus avoided. If water does not move out of the cell sufficiently, intracellular ice crystals can be generated which can cause damage to the intracytoplasmic contents and cell membrane.
4. With the efflux of water from the cell to the surrounding media hyperosmolar state is created in the cells. This will now lead to movement of permeating cryoprotectants in the cell to balance the osmolarity imbalance.

FREEZE INJURIES

- a. *Ice crystal formation:* Extracellular ice crystals (ice spicules) -compress or lacerate the cell membrane and may damage the Zona Pellucida.
Intracellular ice formation is usually lethal as it can damage the blastomeres, can cause spindle damage in oocytes and may damage the intracellular contents.
- b. *Osmotic Stresses:* In case cells are completely dehydrated due to very high concentrations of electrolytes/ cryoprotectants, increased osmolarity of the cells may damage the cellular contents. Such situations may destabilize the cell membranes where rearrangement of protein and lipids constituents may occur, changing its properties.
- c. *Temperature effects—super cooling:* Ice formation releases latent heat of fusion and can raise the temperature of cells. Proteins and lipids may be annealed.

d. *Temperature effects – Cooling rates:*

- Intracellular ice may form if rate of cooling after seeding is too fast. Here it is important that enough time is given to the cells and the cryoprotectant containing media to equilibrate. This process is known as soakage or the holding period. The larger the cell volume, more water content and thicker the container, more should be the soakage/holding period.
- Very slow cooling rate may lead to excessive dehydration of the cells leading to osmotic stresses and their desiccation and collapse, following morphological damage.

EVENTS DURING THAWING

1. In this period change of water phase is taking place. Ice is melting with recrystallization occurring in intracellular and extracellular compartments.
2. With the melting of extracellular ice crystals in the solution due to increase in temperature during thawing there is reduction in the osmolarity of the surrounding solution due to its dilution. Simultaneously there would be comparative increase in osmolarity of the cell due to presence of influxed cryoprotectants. This leads to water influx in the cell from the extracellular compartment.

THAW INJURIES

Intracellular Ice Crystals

- Intracellular ice can form due to insufficient dehydration during the freezing process. Ice formation can also occur if the cells are not thawed quickly and there is time for the water recrystallization to occur.

Osmotic Stress

- Excessive influx of water due to osmotic difference can cause cell to swell and rupture.
- If cells have been cooled very slowly till -80°C and thawing is done rapidly, the cryoprotectants driven into the cell during cooling cannot diffuse out. This can lead to osmotic stress due to the presence of high concentration of cryoprotectants intracellularly.

Gas Expansion

Following the formation of ice in an aqueous solution, all other physical parameters of the residual unfrozen solution may change, e.g. the soluble gas content increases both intra and extracellularly resulting in the formation of gas bubbles. These expanding gases especially carbon dioxide can form bubbles within the cells causing cell disruption and cell membrane destabilization.

Risk of Storing Biological Materials at Low Temperatures

At -196°C , the temperature of liquid nitrogen, it is possible to keep eggs or embryos without loss of viability for decades. However, recently it has been demonstrated that infectious agents such as viruses can spread through liquid nitrogen. Viruses are highly resilient and if straws, vials, or ampoules break while in liquid nitrogen storage any viruses contained within it may contaminate the liquid nitrogen itself. This is known to have caused the transmission of hepatitis. The risk of transmission is significantly reduced or eliminated by alternative storage methods including storage in the vapor phase of liquid nitrogen, or storage in -140°C to -150°C biological freezers.

CONCLUSION

Before beginning a human embryo cryopreservation program, consideration needs to be given to the establishment and running costs, record keeping, patient consent forms, storage levies, and storage times.

1. Costs may include the purchase of a biological freezer, storage tanks, protective mask and gloves, and an appropriate floor covering, resistant to liquid nitrogen spills. Running costs include liquid nitrogen and its delivery fees.
2. It is important to ensure that an appropriate record keeping system is in place before any embryos are frozen. It is also essential that all straws, ampoules, vials, goblets or canes are clearly labeled (with water-insoluble markers) and their positions in the storage tank recorded.
3. For human oocyte or embryo freezing, patient consent forms approved by the governing institution are generally required. These consent forms should deal with issues such as disposal of embryos following the dissolution of the marriage or death of one or both partners.

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