REVIEW ARTICLE

A Review on Cryoprotectant and its Modern Implication in Cryonics

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Abstract

Cryoprotectants are basically some chemical compounds which prevent cells or tissues from damage due to freezing. Mostly, verification and thawing process is used in cryopreservation. The various animal tissues, organs, lymphocytes, cartilages, bone marrow, and proteins can be stored using proper cryoprotectants. Optimization of cryoprotectants is a challenging task, as elevated concentration may cause cytotoxicity. In this article, we discussed the implication, challenges, and recent advances of cryoprotectants along with its types. The importance of cryobank system and its importance were also being emphasized in this article.

Key words: Cryopreservation, vitrification, cryoprotectants, cryobank system, dimethylsulfoxide, glycerol

INTRODUCTION

It has always been a difficult task to preserve and store living organisms, plant tissues, cell lines, yeast, bacteria, fungi, animal tissues, algae, amino acids, lipid based formulations such as solid lipid nanoparticles and so many biological materials fora long period. With this challenge, Polge and his team did a splendid discovery.[1] On 1949, they found glycerol could be used to preserve successfully spermatozoa cells for a long time. Since then, so many scientists have started thinking about further studies on cryobiology and its futuristic importance. In further studies, scientists give importance to vitrification[2] (a process at which cell is stored in freezing conditions for a long time without forming solvent crystals, which could usually cause cryoinjury) and slow freezing process. Due to the rapid acceptance of verification and slow freezing process, a maximum number of animal germplasms, tissues were started to store by these two processes. Further advancement embarks the idea to preserve tissues bellow −130°C to −196°C, which is basically a sub-zero temperature using some substances. It was also observed that all the associated problems of refrigerating storage such as integrity of living cells while storing, freezing injury, forming ice crystals which eventually damage stored cells, could be restricted from using such substance. These substances are also helping in moisturizing the living cells by penetrating inside of stored cells without altering or creating any lethal effect to those cells; such substances are called as the cryoprotectants or the cryopreservatives.[3,4] Without cryoprotectants at low-temperature biological cells damaged, and at in transition phase such as changes from frozen solid to a liquid by a general warming,[5] cells loses numbness, and stiffness, this condition is called thawing. Mostly, slow freezing is preferable for preserving cells, in which cells are frizzed slowly bellow its freezing point, but at a certain point, the formation of spherical crystals can destroy cells chronobiology. These crystals are called as unfrozen fractions.[6] Using cryoprotectants along with some electrolytes and sugars, it is possible to increase the osmotic strength of the solvent by which positive efflux of moisture toward the cells occur, and during these process chances of intercellular ice, the formation becomes negligible. Further cooling causes an increase of viscosity, and due to this, chances of formations of ice crystals would become very infinitesimal. In the last stage of slow cooling, remaining unfrozen fraction transformed to amorphous solids without

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ice crystals. The main objective of cryopreservation is to create a dehydrating environment for preserving cells. In this process, cytosol observed less injury during cold freezing within liquid nitrogen, as numbers of crystalline substances formation would be very minimal. Mostly, it was found that cryoinjuries occur during anomaly of instantaneous super freezing of cells. Slow freezing with lucid cryoprotectants causes fewer injuries.[7] Eventually, cryoinjuries occurs during pre-freezing and post-thawing, within the temperature of 0-40°C. During cryopreservation technique, pH, osmotic pressure, and concentration of cryoprotectant have to be maintained, as elevation can cause cyto injuries. In some cases, if the cell cytosol contains specific fatty acids, i.e., spermatozoa plasma membrane, it helps to maintain proper osmotic pressure and resist shock during thawing and freezing, by which fatty acids help to maintain cells rehydration.

Freezing cells and cryoprotectant

Cryoprotectant function is a string phenomenon as it is a complete mystery, how it works. Functionally, cryoprotectant protects and preserves stored cells by protecting it from frizzed needles and dehumidification.[4] During freezing of cells, probably two possible outbreaks were recorded. One is mechanical damage in cell outer surface is palpable and second is the elevation of osmotic pressure due to chemical and residual unfrozen water in between ice crystals [Figure 1].

Postulated mechanism of cryopreservation

After mixing properly with water, cryoprotectant decreases the melting point of water. This antifreeze or cryoprotectant is such as propylene glycol and dimethylsulfoxide (DMSO). Mostly, 5-15% concentration of cryoprotectant concentration has to be maintained during freezing and thawing of isolated cells. Freezing of cells decays when preservation taking place with liquid nitrogen and cryoprotectant, which results in the formation of large vesicles and1 fewer salt crystals. This process helps to protect cells from any mechanical injuries.

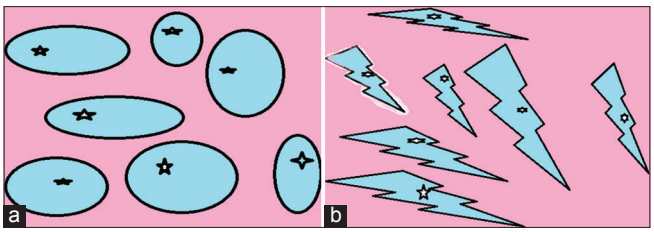


Figure 1: During freezing with cryoprotectant cells retain its shape and structure. (a) While in slow freeze-drying, (b) without cryoprotectant, cells got shrieked and squeezed, the formation of crystals and salt layer in between unfrozen liquids, due to which cells damage occurs[8]

Slower, further formation of relatively large vesicles and fewer salt crystals helps to protect cells from any mechanical injuries. As per evidence, cryoprotectants usually do not form any hydrates, and they are relatively less toxic to cells in elevated concentration. Vast, expensive use of glycerol as cryoprotectants becomes a stepping stone toward finding more than hundred more cryoprotectants within 50 years. In new age penetrating cryoprotectants were using vastly in the pharmaceutical industry because they are having a molecular mass of fewer than 100 Dalton, and they penetrate inside of the cells and maintain moisture during freeze drying.[9]

Vitrification

Organs are most venerable for freezing related damage; they are mostly organized and have less chance to form sequester ice pocket formation compare to tissue suspensions. For functionalize organs after freezing, almost all the parenchyma cell to small blood vessels has to be survived in large numbers after freezing. The simple cryopreservation is not good enough to tackle this problem. A famous cryobiologist Dr. Gregory Fahy, in 1984, suggested vitrification as an alternative to prevent tissues or organs from freezing damage.[10] In cryobiology, vitrification means rapid freezing or transforming into a glass. In this process, large organs were loaded with a large concentration of cryoprotectant before cooling. After cooling, the organs would remain stored intact within the glass pocket. By fight shy of mechanical disability caused by ice and maintaining proper proportions of salts and other molecules, vitrification slowly becoming a lucid process in cryopreservation.

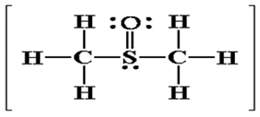
Toxicity emerges from cryoprotectant

In cryopreservation technique, almost half of the portion of the cell is replaced by cryoprotectant molecules. It is really an awful phenomenon when cell contents are replaced by cryoprotectant molecules, but still, cells can survive for long. However, in some cases, cryoprotectant causes toxicity during a freezing point, for example, at warm temperature propylene glycol shown less toxicity compare to ethylene glycol (EG), but in an around zero degrees centigrade, EG would become less cytotoxic than propylene glycol. On the other hand, lipophilicity of cryoprotectant can cause savior toxicity. Another theory suggested that strong hydrogen bonding possibly disrupts hydration shell around macromolecule, due to this toxicity may occur. Sometimes, polarity of cryoprotectant causes cytotoxicity. Toxicity can also be reduced by adding combinational cryoprotectants, for example, DMSO and formamide combination reduces cytotoxicity.[7,11,12]

SOME COMMON CRYOPROTECTANTS USED IN PHARMACEUTICAL PRESERVATION[13]

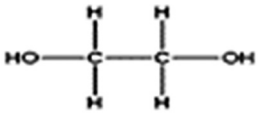
DMSO

DMSO is basically an organosulfur derivative. The molecular formula is $(CH_3)_2SO$. This colorless solution can able to dissolve both polar and non-polar compounds.



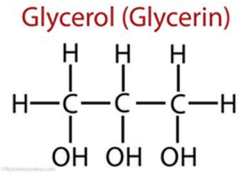
DMSO has typical properties; it freezes within 18.5°C. That means, bellow room temperature DMSO transformed into solids, and this property makes it most suitable for cryoprotectant.

EG: EG alters the hydrogen bonding when it mixes with water. Purified EG has a freezing point at about −12°C; but after mixing with 40% water and 60% EG, the freezing point of the mixture would depress and mixture becomes incapable of forming crystalline substances. This condition leads to a transformed freezing point at −45°C. This property of EG makes it the most effective candidate for cryoprotection.



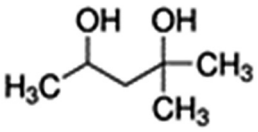
Glycerol

The colorless, odorless, viscous liquid of simple polyol (sugar alcohol) compound named as glycerol or glycerine. Glycerol has good kosmotropic properties; it forms hydrogen bonds with water molecules. This condition makes difficult to form ice crystals by mixture (70% glycerol and 30% water), unless and until the temperature is very low such as −37.8°C. Compared to another cryoprotectant, glycerol is less toxic in high concentration.



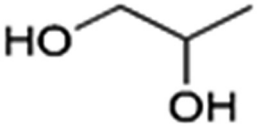
2-Methyl-2, 4-pentanediol (MPD)

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



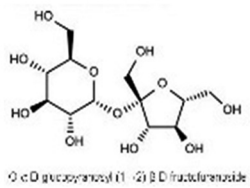
Propylene glycol

It is widely used as aircraft deicing fluid. Propylene glycol is sold under the name of RV or marine antifreeze. It also carries the property of automotive antifreeze.



Sucrose

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



Some commonly used cryoprotectant, extenders, and co-cryo protectant used in cryopreservation studies depicts in Table 1.

Table 1: List of various cryopreservatives

Serial number	Name of cryoprotectants	Serial number	Name of cryoprotectants
1	Acetamide	16	Pyridine-N-Oxide
2	Albumin	17	Propylene glycol
3	Ammonium acetate	18	Ribose
4	Choline magnesium chloride sodium bromide	19	Serine
5	Diethyl glycol	20	Sodium chloride
6	Dimethylacetamide	21	Sodium bromide
7	Dimethylsulfoxide	22	Sodium iodide
8	Ethanol	23	Sodium sulfate
9	Erythritol	24	Sorbitol
10	EG	25	Sucrose
11	Glycerol	26	Triethylene glycol
12	Glucose	27	Trimethylamine
13	Formamide	28	Acetate
14	Glycerophosphate	29	Xylose
15	Proline	30	Valine

TYPE OF CRYOPRESERVATION

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

Isobaric cryopreservation

Isobaric cryopreservation technique deals with the preservation of cells in 1 atm osphiric pressure. This process is widely used, but the certain limitation of cyto cellular damage makes it more bizarre.

Hyperbaric cryopreservation: By increasing elevated pressure, it reduces temperature to sub-0 celsius. This condition leads rapid freezing with intact biological tissues. By which, many tissues can be stored by this process, i.e., kidney (10000 atm) cells (200 atm) and liver (70 atm).

OPTIMIZING FREEZING

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

If cells were cooled very slowly and steadily, then extracellular fluids of the cells form ice crystals, which pinch the cells to withdraw all intracellular components. At elevated -350°C temperature, cells can lose its structure and shape. On the other hand, rapid cooling can cause serious damage intracellular integrate of cells, where cytosol, parenchyma, nucleus, and almost all cellular component rapidly freeze which causes intracellular needed formation. All the integral parts of cells may come out, and the cell may die due to shrinking.

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

Carrier solution or base perfusate used in cryoprotection

Carrier solution acts as a life support or buffering substance for preserving cell. The main purpose of carrier cell is to maintain life integrity in cells during near freezing condition. Carrier solutions constituted with some essential buffer, osmogens, nutritional elements, salts, and certain apoptosis inhibitors. To maintain stability within the cells, carrier solution maintains iso-osmolarity, which makes cells not to lose its original shape. As for example M22, cryoprotectant solution is used with specific carrier solution is called as LM5. Most importantly carrier solution concentration cannot be altered with altering cryoprotectant concentration. It should remain constant, based on the cells to be preserve.[15]

Ice blockers: Ice blockers are the substance which prevents ice to grow. While temperature is low and pressure is high, this ice blocker binds with contaminants or ice inculcator to cleave ice formation. Examples are low molecular weight polyvinyl alcohol, polyglycerol, called as X-1000 and Z-1000. Mostly, ice blocker is used in vitrification process.[16]

RECENT ADVANCES IN CRYOPRESERVATION

Due to recent advancement of organ and tissue transplantation, it is become important to have cryoprotectant research. Wang *et al.*,[17] did some extensive research on preserving ovarian tissues. He used 0.1 M fructose and DMSO to preserve seven rat ovaries. It takes over 30 min to freeze and stored in liquid nitrogen overnight before thawing. Amir Arav's group (2005)[18] develops a novel technique using 1.4 M DMSO (10% by volume) for preserving ovaries. This device transplanted in a test tube where freezing of organs can be possible between 0 and -35°C. This device far end is super cool and stable. The organs which are about to preserve at first must be freeze in liquid nitrogen than exposed and thawed to 68°C for 20 s than 37°C for 2 min. In this process, preserved organ shown immediate blood perfusion after transplantation. Dittrich *et al.*[19] developed a method by freezing pig uteri in near dry ice temperature. It was tried to prove that this process is most efficient then vitrification technique. Berejnov *et al.*[20] studied the effect of cooling rate on vitrification of aqueous solution. It was observed that T = 295 k to T = 77 k temperature vitrification is reported in liquid nitrogen for almost all the cryopreservatives. It was concluded and confirmed that by X-ray crystallography; the transition from polycrystalline to vitreous occurs within the span of 2% w/v in cryopreservatives. By which polycrystalline ice from hexagonal to cubic depends on the elevation of cryopreservatives cooling rate and concentration. Anderson

et al.[21] studied about ovarian cryopreservation for fertility preservation. They concluded that oocyte cryopreservation technique has more advantages as it produces approximately 100 fold more babies as it can preserve a large number of oocytes within primordial follicles without further hormonal therapy. Chong *et al.*[22] studied about cryopreservation of neurospheres which is derived from human glioblastoma multiforme. Cryopreservation of neurospheres cried out using 90% serum and 10% DMSO. Further vitrification yields self-renewal and multipotential properties. Xu *et al.* (2010) studied the role of the apoptotic pathway in the low recovery rate after cryopreservation of dissociated human embryonic stem cells (hES). It was observed that recovery of cells after cryopreservation of hES become a huge challenge. It was found that the generation of reactive oxygen species is significantly increased with F-actin altered distribution. Further analysis insists the activation of caspase-8 and caspase-9, which causes an increase in cellular toxicity.. Cetinkaya and Arat[23] studied about the bio-banking system of cryopreservation for cartilage and tissue cells. Vitrification is used to preserve primary adult cartilage and fatal cartilage cells. The cartilage cells were slowly freeze using EG, Ficoll, and sucrose. During slow cooling, three different cooling rates were set as 0.5, 1, and 2°C/min. Results astonished all by no significant changes in viability rations, proliferative activity, and glycosaminoglycan synthesis observed after vitrification (1°C/min). This study emphasizes the needs of vitrification in bio-banking of cell donor sources in nuclear transfer studies. Wong *et al.*[24] did vital studies on human embryos cryopreservation and its fertilization rate. In modern days, cryoprotection human embryos cells are routine work, but the optimizing success rate of transplantation is a matter of question. In this study, high quality randomized trials were used to find best cryoprotectant protocol for fresh transfer of embryos. Alexandre Rodrigues Silva *et al.*[25] reviewed about mammalian conservation biology and cryopreservation. They enlightened the importance of germplasm cryopreservation of embryos, gonadal tissues, gametes, and some somatic tissues. They also dignified the importance of more usage of cryobanking for the preservation of cells and tissues of threatened species.

CONCLUSION

Cryobiology will have a significant impotence in near future as a number of transplantation of vital organs of humans are becoming more demanding in the modern age. Yet, so many important complications are to be mitigated such as the exact mechanism of cryoprotectant, mode of action, and cellular interactions with potential toxicity. More extensive research is needed in respect of optimizing cryoprotectant concentration and slow and high freezing process. It was observed that macromolecular cells could easily preserve by cryoprotectant, but as far as large tissues or organs (kidney, liver, heart, lungs, and skin tissues) are concerned; the success rate of freezing is not up to the mark using cryoprotectant. Modern emerging cryobank is a promising approach toward organ transplantation.

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