



## **Cryopreservation Technology**

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### **Abstract**

This paper is about cryopreservation technology is a well know process currently use in the medical field in order to preserve the cells, tissues, organs and other biological samples for long time in ultra-low temperature. Word “Cryo” comes from Greek language which represent the ice cold or frozen “Preservation” is the process of keeping save or alive for long period of time. Cryopreservation is basically done under liquid Nitrogen which maintains the temperature at -196 °C without disturbing to the viability of the cells, tissues or organ. Stem cells which are mainly used in drug discovery process and I the organ transplantation process we have to keep those testing samples and organs alive for long period of time and due to the ice crystal formation, disrupting of cell membrane, osmotic pressure are the main possible reasons can caused to destroy the cell viability during the freezing. These injuries occur during the freezing is commonly use to called as cryoinjury and it can be avoid using cryoprotectants and proper temperature controlling. This paper contains the basic process of cryopreservation along with the technique behind the process.

Key words – Cryopreservation, Cryoprotectant, Cryoinjury, Thawing

### **1. Introduction**

Cryopreservation is a technique which can use to conserve/ preserve the cells, tissues, extracellular components, cell organelles and organs for long time in order to further usage. This can be achieving by exposing those cellular components at very low temperature using solid Carbon Dioxide or Liquid Nitrogen. Cells, tissues and organs which are used to store for further researches

can't store long time using simple cooling or freezing techniques due to the formation of ice crystals cells can be cause to death. At the low temperature biological and chemical reactions in the living cells will reduce gradually due to the low temperature and freezing become fatal to many organisms due to the formation of ice crystals in intracellular and extracellular parts of the cell. So in cryopreservation we have overcome the hardest part which is transition of water in to the ice crystal inside the cells. Cryopreservation technique is based on the ability to enter certain molecule in to the cell in order to prevent the dehydration and intercellular ice crystals formation which will cause to destroy the cells and organs during freezing. Those molecules are called as Cryoprotective agents (CPA).

Cryopreservation technique is not a new thing and it is naturally occur in many animals like Water bear, Wood frog, and in Turtles. Water bear is a multicellular microorganism which can survive in low temperature by replacing internal water with sugar Trehalose which cause to the protect cell membrane. Wood frog also an animal who can manage the freezing of their blood and cells by accumulating urea in their tissue and liver glycogen which turns to glucose to prevent from water crystallization. Both of these urea and glucose considered as "cryoprotectants" which cause to limit the amount of crystal formation and reduce osmotic shrinkage of cell.

## 2. History

In 1949 Scientist Ernest John Christopher accidentally got to know the cryoprotective properties of glycerol on sperm so he is considered as the first person who discovered a method to preserve living cells and tissues in very low temperature. Then Ploge experimented about the pregnancy rate of cattle's using the sperms preserved for one year and this experiment cause to lead the path to artificial insemination in future. Later in 1953, Scientist Jerome K. Sherman from University of Iowa experimented about the successful method to preserve human sperm using freezing technique and he founded the world's largest sperm bank. The term cryobiology was origin in 1964; as cryo mean cold, bio is life and logos are science. So the cryobiology is the biological activity affects at low subzero temperature and cryopreservation on the range of  $-80^{\circ}\text{C}$  to  $-196^{\circ}\text{C}$ . Later in 1983 Alan Trounson an Australian biologist achieved the pregnancy after freezing human embryo after the three days of fertilization and in 1986 Christopher Chen abled to preserve human oocytes successfully. In 1953 James Lovelock realized the damage occur to the red blood cell during freeing is occur due to the osmotic stress and it cause to the increasing the salt concentration dehydration cells and in the mid of 1950 he experimented about the cryopreservation of rodents. Application of cryopreservation to the human is begun with 1954 and in 1963 Peter

Mazur at Oak Ridge National Laboratory in U.S described that the lethal inter cellular freezing can be avoid by cooling it slowly by allowing the water to leave the cell. And he found that cooling rate at 1C/min is necessary for mammalian cells after the treatment with cryoprotectants like glycerol. The first human body use to be frozen for future investigation was done in 1967 that died due to the cancer “James Bedford”; an American psychology professor.

### **3. Cryopreservation medium**

In the cryopreservation process cells, tissues and organs are store at ultra-low temperature of liquid nitrogen (-196°C). In this temperature those cells enter to the “ABSOLUTE QUIESCENCE” state where physical, biological and chemical reactions are stopped for the conservation time. Dry ice is another component can use along with those biological samples to freeze, in order to gain extreme cold temperature for preservation. Dry ice is the solid state of carbon dioxide gas and it is consider as a mobile coolant and can be store in Styrofoam boxes. This is mainly used for transport organs, biological samples and tissues.

Main problem occur in cells during the low temperature is transition of water phase in to the ice and the fast cooling process result to the intracellular ice lattice formation while slow cooling cause to the osmotic changes in the cell due to the interactions between the intracellular and extra cellular solution and the damage occurs due to the above activities in the cells during the freezing is known as “Cryoinjury”. Cooling and thawing of cells result to alter the physical, chemical and biological reactions and as a result of solution’s concentration between intracellular and extracellular components become varies. Due to that the osmotic pressure become imbalance and causes to rupture the cell membranes and as a result of that intracellular and extracellular solute particles fond to create ice lattice in the cells resulting to the cryoinjury. There is a standard optimal freezing point for each cell to withstand for the cryoinjury and to recover the maximum amount of cells by thawing and this is achieved by minimizing the amount of ice crystals formation in the cells by many techniques like freezing in low speed allowing intercellular water to leave without forming ice crystals, using croprotectant to sequester water and by thawing rapidly to avoid regeneration of ice crystal growth.

Most of the times in cryopreservation process biological samples subjected to the cryogenic temperature for the preservation of cells and it is depend on the ability of some components to enter to the cell and avoid the formation of ice lattice which are cause to the dehydration and cell death. Those components which can be entering to the cells in order to avoid cryoinjury are called as Cryoprotective agents. Trehalose is a natural cryoprotector occur in the body of organisms which give

a support to survive in dehydration by stabilizing the cell membranes and mainly use for the preservation of sperm, stem cells and blood cells in the body.

Cryoprotectants are the macromolecule substances which are used in freezing medium to protect the cells from the formation of intracellular ice crystal formation during the freezing process and thawing process. When selecting a suitable type of cryoprotectant we have to consider following characteristics like, high solubility, low freezing point, and low toxicity at high concentration, low molecular weight and capability to protect cell membrane during cryoinjury.

Dimethyl Sulfoxide (DMSO), Propylene glycol (PG), Ethylene Glycol (EG) and Glycerol (GLY), Dextran, BSA and Serum are commonly using cryoprotectant reagents and Glycerol is mainly use as a cryoprotector for red blood cells, Ethylene Glycol is preferred to use llama embryo cryopreservation while DMSO is mainly use for other cells and tissues. Above cryoprotectants are mainly two types as Permeating cryoprotectants and non-permeating cryoprotectants.

Table 1. Types of cryoprotectants

<b>Permeating cryoprotectants</b>	<b>Non permeating cryoprotectants</b>
Intracellular cryoprotectants	Extracellular cryoprotectants
Lower the freezing point of the solution	Have two types as low molecular weight CPs and High molecular weight CPs.
Increase the viscosity by reducing diffusion in solution Ex: Glycerol, Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG)	Low Molecular weight CPs : sucrose, maltose, trehalose High molecular weight CPs: Polyvinyl Pyrrolidone (PVP), Dextran, Serum

Action of cryoprotector occurs as those cryoprotectors have capability to lower the melting point of water when they dissolve in water. Those cryoprotectors are often known as “antifreeze” as they can lower the freezing temperature by increasing the viscosity. 5% to 15% concentration of cryoprotectors is necessary to the survival of isolated cells and tissue after freezing in the liquid nitrogen temperature. Without cryoprotectant all the water volume in the cells become freeze during the cooling. When cryoprotectors becomes concentrated in order to make melting point equal to the surrounding temperature ice grow in the cells will stop.

At -100°C temperature unfrozen remaining liquid in the cells turns to a glass which allow to preserve for long time. If the starting cryoprotector concentration is high the unfrozen volume will be high in volume. Basically cryopreservation on depend on two main concepts, the dehydration of

cell using osmolytes and prevention of ice crystals formation using cryoprotectants. Sugars like sucrose cannot transport through the cell membranes without transporters present in outer cell membrane, when the extracellular concentration increased the intracellular water moves out from the cell resulting the dehydration of cell. This cause to reduce the intracellular water in the cells which are available to form ice crystals. The ice crystals formation in the cell can be reduce by addition of cryorotectants like DMSO and methanol. Those compounds penetrate through the cell membrane due to their less molecular mass and low hydrophobicity cause to disrupt ice crystals formation.

#### **4. Methodology**

Cryopreservation technique is basically depend on two main concepts which are; dehydration of cells using osmolyte substances and prevention of the formation of ice crystals during cooling process. This process has 7 main steps as follow; considering the regeneration of plant cells.

##### **1. Plant material selection**

Mainly there are two factors we should consider before selection a plant material; they are the morphological and physiological condition of the plant material because those materials should have able to survive during the cryopreservation process. When considering the nature of cells should be far as possible from meristematic state. Basically the young, small calls which are highly vacuolated cells are better for the cryopreservation. But in some species highly embryonic cells are better than non-embryonic or poor embryonic cultures as they do not show regrowth after the thawing. So high density tissues meristem, embryo, ovules, seed can be used.

##### **2. Addition of cryoprotectant**

Cryoprotectants are the agents which can use to reduce the cryoinjuries which is the process of formation of ice crystals inside the cell cause to rupture the cell wall.

- Intracellular cryoprotectants – Glycerol, dimethylsulfoxide
- Extracellular cryoprotectants – Polyvinyl pyrodine

Normally two cryoprotectans have to add together in order to be more effective and increase the efficiency.

- Dimethylsulfoxide is a polar aprotic solvent which can be dissolve in both polar and non-polar solvents and very low cost, less toxic compound. It has ability to reduce electrolyte concentration in the cells during cryopreservation and freeze in 18.5 °C.

- Glycerol is colorless, odorless liquid and has ability to form hydrogen bond with water molecules resulting to reduction of ice crystals formation in cells till  $-37.8\text{ }^{\circ}\text{C}$  but this is a toxic compound.

### 3. Freezing

This is the main step in the cryopreservation. The success of the preservation is depending on the freezing techniques. But in this stage there may be many damages occur, like dehydration extracellular ice formation, intracellular ice formation result to the death of cells.

- Dehydration is occurring due to the migration of water out of the cell and it lead to the formation of extracellular ice formation also.
- When ice crystals forms in the water the solution effect may occur due to the concentration of solute particles in the remaining water of the cell and this high risk concentration of solution cause to the rupture the cell wall and membranes. To avoid those damages can be occur during this process there are many methods following by the researchers.

Slow freezing is also known as slow programmable freezing (SPF). This method was initiated in 1970 by freezing the human embryo. Samples like oocytes, blood, embryo, sperms, stem cells and tissues can be preserving by this method by allowing to cool slowly permitting to sufficient water and freeze at rate nearly  $0.1\text{-}10\text{ }^{\circ}\text{C}/\text{minute}$ . Here the cells and tissues are partially dehydrated for the better survival and in some cases extracellular crystals can be form but intracellular crystals will not form. Biomaterials like polyvinyl alcohol can be used to reduce the formation of ice crystals along with cryoprotectants.

Vitrification is the process introduced by Greg Fahy in 1980s, which will able to cryopreservation the samples without damage due to the ice crystal formation. This method is mostly used in clinical cryopreservation and this need the addition of cryoprotectant before cooling. The vitrification of water is promoted by the rapid freezing and it need to increases the viscosity and decreases the freezing temperature.

In Rapid freezing materials are subjected to freeze quickly at  $-25\text{ }^{\circ}\text{C}$  or below that, ice crystals form at this step is very small so it will not cause to damage the cell structure. Very simple and easy and due to the speed have least chance to development of intracellular crystals. Here samples need direct contact between straw and the nitrogen vapors for 8-10 min in  $-196\text{ }^{\circ}\text{C}$  nitrogen. Persufflation also a method that use a noble gas like oxygen in order to replace with blood in the organ helps to cool organs quickly.

Step wise freezing is use liquid nitrogen. Initially temperature drops down to the -20 °C to - 40 °C and allows freezing and then freezing is stopped for 30 minutes and again rapidly freeze in liquid nitrogen.

#### 4 . Storage

In this step we have to maintain the cells and tissues which we are preserve in a specific temperature. This storing temperature must be having an ability to stop metabolic activities and biochemical activities; so the best option is liquid nitrogen at -196 °C. In this process to avoid the damage should supply liquid nitrogen continuously.

#### 6. Thawing

After the storage step those vial are transfer in to the elevate temperature, transfer in to a water bath containing water at 20 °C – 25°C. This will result to avoid the ice crystals formation rapidly. In this step spraying of alcohol vial is result to avoid the contamination.

#### 7. Washing and Re- Culturing

The preserved and thawed materials should wash few times in order to remove the cryoprotectant materials and reculture in newly prepared medium. If we used the low toxic ot non-toxic cryoprotectants it is not necessary to wash.

#### 8. Measurement of viable cell and Regeneration of plant

Due to the stress subjected by the cells, in some case cells can be dying. So calculate the amount of viable cells using the formula  $\frac{\text{No of cell Growing}}{\text{No of cells thawed}} \times 100$

Finally those cryopreserved material can be used for the regeneration of the desired plant. For the better result it should be done carefully and also have to maintain appropriate environment conditions which are favorable for the better regeneration of plant.

### 5. Conclusion

Cryopreservation is a frequently using technique in medical technology and its applications spread in wide areas. Cryopreservation of organs and cells is use in currently as organ transplattation is the most effective treatment for the patients who are having end stage organ failures. So in medical field they use to store donor's organs in preservation mode using protectants and HLA matching to increase the storage time for the long term survival of the organs. So here also while cryopreservation is doing the cryoprorecting agents, freezing and thawing process is do as same as above mention procedure. Mostly hearts and kidneys are preserving using this method. In cryosurgery this is the method of cooling use to extreme cool to destroy abnormal cells and tissues in the organs. Mostly use liquid nitrogen and argon or carbon dioxide also can be used. Liquid

nitrogen has  $-196^{\circ}\text{C}$  which have ability to cool any contacted cells instantly. This method is mostly use to treat in cancers. Seed banking also use cryopreservation technique in order to store seed for long time. These seed banks are the most appropriate way to conserve plant out from their natural habitat, and they use to conserve the seed which the plants are extinct from the world, endemic, economically important and the plants having medicinal value for the utilization in future. This method is also considered as ex situ conservation and it is done by using liquid nitrogen. There the seeds are dried up to 5% and then frozen in  $-20^{\circ}\text{C}$  and in cryopreservation they use to remove the embryo of the seeds and then freeze by using liquid nitrogen at  $-196^{\circ}\text{C}$ . Gene Banks are biological material collection center where use to preserve genetic materials like plant DNA, animal sperms and eggs. There those specimens collects and use to preserve in  $-196^{\circ}\text{C}$  in liquid nitrogen. In plants can preserve by cutting from the plant or stock as seeds and in animals sperms and eggs can freeze in zoological freezers. Freezing and storage of hematopoietic stem cells which are present in bone marrow and blood peripherals also an advance application of cryopreservation technique. There the stem cells which are collected from the patient before the chemotherapy are cryopreserved to use after the chemotherapy. Those cryopreserved cells are thawed and infuse again to the body after the chemotherapy as high dose of chemotherapy is toxic to the bone marrow.

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