S. S. College, Jehanabad

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Topic: Cryopreservation of cells, tissues and organs - Part 1

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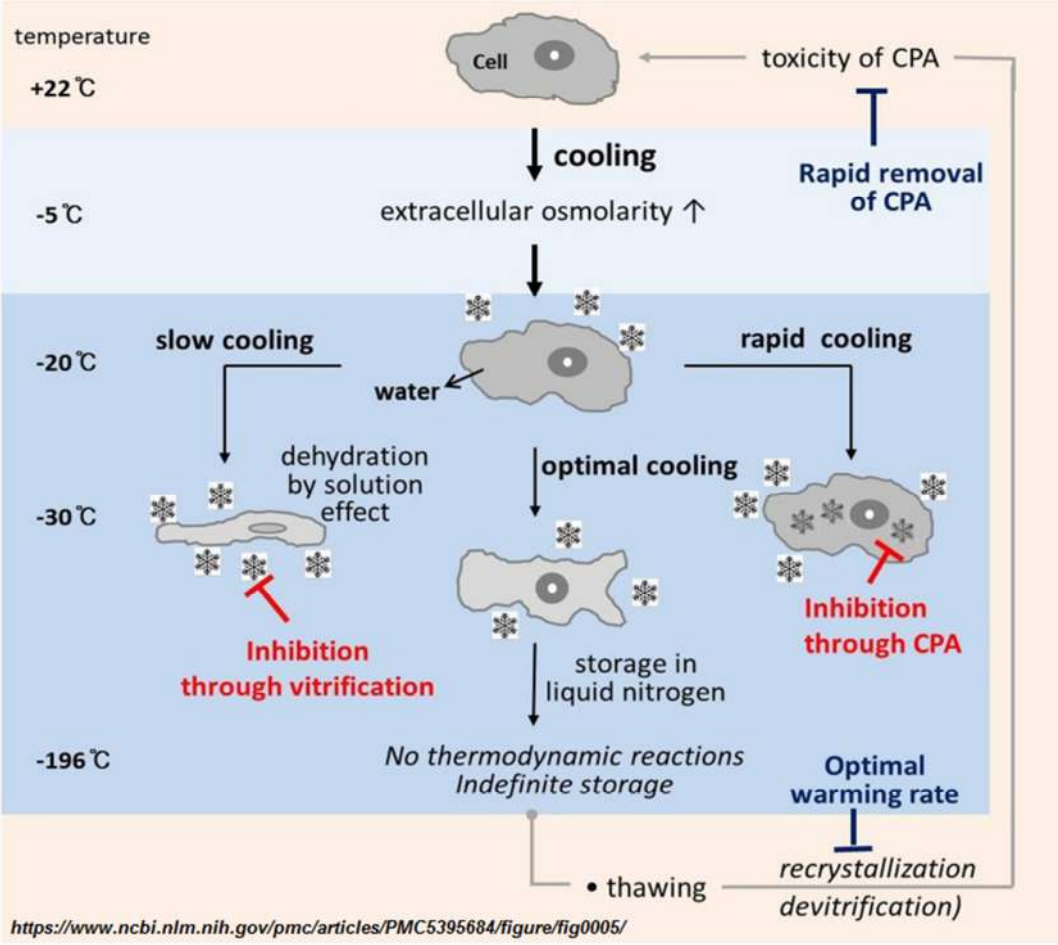
CRYOPRESERVATION OF CELLS, TISSUES AND ORGANS – PART 1

Cryopreservation is the process whereby biological specimen are preserved at subzero temperature. It is based on the fact that all biological and chemical reactions in living cells are dramatically reduced at low temperature. However, freezing of a biological specimen to subzero temperature is fatal to most living organisms, since major constituent of all biological materials is water and upon freezing this water transforms into ice crystal at both intracellular and extracellular level that results in changes to the chemical settings of cells, and loss of integrity of cell architecture and tissue organization. Intracellular crystals can damage the cell walls and structure, while the extracellular precipitation of water as ice crystals increases the salt concentrations to levels that can cause damages to the cells. Therefore, the formation of these ice crystals must be controlled during the temperature based preservation of biological materials. The major hurdle for cryopreservation is the overcoming of water-to-ice phase transition at low temperature. Fast cooling leads to rapid intracellular ice formation that causes cell injury, while slow cooling causes osmotic changes due to the effect of exposure to highly concentrated intra- and extracellular solutions between cells and the extracellular ice as shown in figure below.



Physical events and injury of cells during cryopreservation as a result of freezing

Therefore, a **cryopreservation technique** deals with the freezing behavior of cells and its alteration in the presence of a **cryoprotective agent (CPA)**, also known as **cryoprotectant**. A

cryoprotectant affects the rates of water transport, nucleation (first step of ice crystal formation) and ice crystal growth. However, unlike in single cell suspensions, bulk tissues have different heat and mass transfer effects that occur during the cryopreservation of bulk tissues and thus these factors make it more difficult to achieve rapid cooling and warming rates as well as an equal distribution of cryoprotectant. With use of CPA, it is possible to freeze cells, tissue and organs without the formation of ice crystals, a process known as **vitrification** (formation of *vitreum* (Latin) or glass i.e. glass like clear frozen solution in terms of preservation). Cryopreserved cells or tissues possess some advantages for basic research and current and future clinical applications. With the constant availability of cryopreserved cells and tissues, extensive quality testing can be performed to determine the suitability of the cells or tissue for transplantation without the need to obtain fresh samples.

Principles of Cryopreservation

It is the use of very low temperature to preserve structurally intact living cells and tissues for a long period of time. The method of preserving at low temperature varies depending upon the cells types among different mammalian species. It is generally be grouped into te following two types as described above which are **slow freezing** and **fast freezing**. Fast freezing of cryopreservation is also known as vitrification. A comparison of both the methods of cryopreservation is given below (see table).

Characteristic	**Procedure**	
	Slow freezing	**Vitrification**
Working time	More than 3 h	Fast, less than 10 min
Cost	Expensive, freezing machine needed	Inexpensive, no special machine needed
Sample volume (μL)	100–250	1–2
Concentration of CPA	Low	High
Risk of freeze injury, including ice crystal formation	High	Low
Post-thaw viability	High	High
Risk of toxicity of CPA	Low	High
Status of system	Closed system only	Opened or closed system
Potential contamination with pathogenic agents	Low	High
Manipulation skill	Easy	Difficult

Whatever the method is, both the processes enable us to subzero storage of biological materials. The major steps in cryopreservation are following;

- The mixing of CPAs with cells or tissues before cooling;
- Cooling of the cells or tissue to a low temperature and its storage;
- Warming of the cells or tissues; and

- Removal of CPAs from the cells or tissues after thawing

Therefore, appropriate use of CPAs is important to improve the viability of te sample to be cryopreserved.

Cryoprotective agents (CPAs)

A cryoprotective agent is a substance that is used to prevent ice formation that causes freezing damage to the biological materials such as cells, tissues, or organs. It does so by reducing the ice formation at any temperature by increasing the total concentration of all the solutes present in the system. It is generally a fluid and able to penetrate the cells, and have low toxicity. Different cryoprotective agents are used in different cell types and therefore its use is also dependent upon cell type, cooling rate, and warming rate and its concentration are optimized accordingly. The CPAs are divided into categories, which are as follows;

1. **Cell membrane-permeating cryoprotectants**, such as dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol; and
2. **Nonmembrane-permeating cryoprotectants**, such as 2-methyl-2,4-pentanediol and polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, and various sugars.

Unlike synthetic chemical, biomaterials such as alginates, polyvinyl alcohol, and chitosan can be used to impede ice crystal growth, along with traditional small molecules. The direct inhibition of ice crystal formation and application of antioxidants and other compounds have been used to attempt to reduce cell death from processes such as apoptosis during the freezing and thawing cycle. Common CPAs used in the cryopreservation of biological materials are as follows;

Glycerol: It was Polge et. al. in 1949, who discovered the cryoprotective effect of glycerol. This polyol compound remained the most effective of additives until the protective effect of DMSO was demonstrated by Lovelock and Bishop in 1959. Glycerol is a nonelectrolyte and thus may act by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature. It is widely used in the storage of bacteria and animal sperm.

Dimethyl sulfoxide (DMSO): It is a natural chemical compound derived from trees as a by-product from the manufacturing of paper and is composed of two methyl groups and sulfur and oxygen. It was first synthesized by the Russian Chemist, Alexander Zaytsev, in 1866. It has been commonly for the cryopreservation of cultured mammalian cells because of its low cost and relatively low level of cytotoxicity. It acts by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature. However, a decline in the survival rate and the induction of cell differentiation caused by DNA methylation and histone alteration has been reported, which create some difficulties for its use in routine clinical applications.

Polymers: Encapsulating CPAs during cell resuspension is another approach of utilizing cryoprotective agents for better cryopereservation. Among encapsulating materials, synthetic nonpenetrating polymers can provide cryoprotection of cells within the scaffold, thereby bypassing the limitations of diffusion in higher-dimensional cryopreservation. Vinyl-derived

polymers, such as polyethylene glycol ($C_{2n}H_{4n+2}O_{n+1}$, molecular weight: 200–9500 Da), polyvinyl alcohol [$(C_2H_4O)_n$, molecular weight: 30–70 kDa), and hydroxyethyl starch (130–200 kDa), have a capacity to decrease the size of formed ice crystals.

Commonly used cryoprotective agents and their uses			
Cryoprotective agents (CPAs)	**Membrane permeability**	**Possible toxicity**	**Applied in cryopreservation**
Cell Banker series	Yes	Unknown but less than that of DMSO	➢ Adipose tissue-derived stem cells ➢ Amniotic fluid ➢ Bone marrow ➢ Mammalian cells ➢ Synovium
Dimethyl sulfoxide (DMSO)	Yes	➢ Reduction in heart rate ➢ Toxicity to cell membrane	➢ Adipocyte tissue ➢ Amniotic fluid and umbilical cord ➢ Bone marrow ➢ Dental pulp ➢ Embryo (combined with EG or propylene glycol) ➢ Embryonic stem cells (alone or combined with EG) ➢ Hepatocytes ➢ Microorganisms ➢ Oocyte (combined with EG) ➢ Platelet ➢ Teeth ➢ Testicular cell/tissue
Ethylene glycol (EG)	Yes	➢ Gastrointestinal irritation ➢ Pulmonary edema ➢ Lung inflammation	➢ Amniotic fluid ➢ Dental pulp
Glycerol	Yes	Renal failure	➢ Amniotic fluid ➢ Microorganisms ➢ Red blood cell ➢ Spermatozoa ➢ Teeth
Trehalose	No	Relatively less toxic	➢ Adipose-derived stem cells (combined with vitrification) ➢ Embryo (combined with vitrification) ➢ Ovarian tissue (combined with vitrification) ➢ Red blood cell ➢ Spermatozoa ➢ Stem cells (combined with propylene glycol)
Propylene glycol (1,2-propanediol)	Yes	Impairment in the developmental potential of mouse zygotes	➢ Embryo ➢ Hepatocytes

Proteins: Some proteins are also used as cryprotectant for thecryopreservation of biological materials, like sericin. ***Sericin*** is a water-soluble sticky protein (~30 kDa) isolated from the

silkworm cocoon and has been developed as a fetal bovine serum- or DMSO-replacing CPA for human adipose tissue-derived stem or progenitor cells, or hepatocytes. ***Small antifreeze proteins*** derived from marine teleosts or fishes have also attracted attention as CPAs.

Cell Banker series: These are newly developed cryoprotectant solution, commonly termed as Cell Banker and are manufactured largely by Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan. These solutions allow for rapid cell cryopreservation at −80 °C, and have been shown to achieve better survival rates following freezing and thawing compared to other CPAs. They contain 10% DMSO, glucose, a prescribed high polymer, and pH adjustors. Serum-containing Cell Bankers 1 and 1+ can be used for the cryopreservation of almost all mammalian cells. The nonserum-type Cell Banker 2 is optimal for the cryopreservation of cells in serum-free culture conditions. Cell Banker 3 (or Stem cell Banker) is composed of 10% DMSO and other inorganic compounds (US20130198876) and satisfies the criterion of a chemically defined known ingredient that is xeno free and is thus suitable for the preservation of somatic stem cells and induced pluripotent stem cells.

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