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# CRYOPRESERVATION

**WHY PRESERVATION IS IMPORTANT?**

Until two decades ago the genetic resources were getting depleted owing to the continuous depredation by man. It was imperative therefore that many of the elite, economically important and endangered species are preserved to make them available when needed. The conventional methods of storage failed to prevent losses caused due to various reasons. A new methodology had to be devised for long term preservation of material.

**VARIOUS METHODS OF STORAGE**

1. Cryopreservation - generally involves storage in liquid nitrogen.
2. Cold storage - it involves storage in low and non freezing temperature.
3. Low pressure – it involves partially reducing the atmospheric pressure of surrounding.
4. Low oxygen storage - it involves reducing the oxygen level but maintaining the pressure.

**CRYOPRESERVATION-**

It literally means preservation in "frozen state." Cryo is Greek word (krayos – frost).

**Definition:** Process by which any living cells, tissues, organs or entire bodies are protected from decay by storing them at extremely low temperatures.

It is a process where tissues, organelles, cells, extracellular matrix, organs or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temp (typically -80 degree Celsius using solid carbon dioxide or -196 degree Celsius using liquid nitrogen.

**Aim/Principle:**

- To bring cells or tissue to a zero metabolism and non dividing state by reducing the temperature in the presence of cryoprotectant. At low enough temperatures, any enzymatic or chemical activity which might cause damage to the biological material is effectively stopped.
- Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice during freezing.
- Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed Cryoprotectants.
- New methods are constantly being investigated due to the inherent toxicity of many cryoprotectants. Typically -80 °C using solid carbon dioxide or - 196 °C using liquid nitrogen.

**HISTORY**

1. Early theoreticians of cryopreservation was James Lovelock (born 1919) known for Gaia theory. He suggested that damage to red blood cells during freezing was due to osmotic stress.
2. 1949 – Ernest John Christopher Polge, was a English biologist who solve the mystery of how to preserve living cells and tissues at very low temperatures. He accidentally discovered the cryoprotective properties of glycerol on fowl sperm.

3. 1953 – Jerome K. Sherman was a doctoral candidate at the University of Iowa. His research led him to successfully freezing and thawing human sperm. He founded the world's first sperm bank.
4. 1964 – The term cryobiology was invented. It can be literally translated as : "cryo" = cold, "bios" = life, and "logos" = Science
5. 1988 – Yves Menezo is a French biologist who gave his name to the first commercial culture media used in in-vitro fertilization.
6. 1995 – Edouard Servy and the biologist Zishu Liu were the first in the world to successfully transplant a cryopreserved blastocyst following intracytoplasmic sperm injection**.**

**METHODS OF CRYOPRESERVATION**

- Over solid carbon dioxide (at -79 degree)
- Low temperature deep freezer (at -80 degree)
- In vapor phase nitrogen (at -150 degree)
- In liquid nitrogen (at -196 degree)

**BASIC PROTOCOL/ MECHANISM OF CRYOPRESERVATION**

The cryopreservation technique involves following steps :

1. Selection of material and Cell Harvesting
2. Addition of cryoprotectant and Media preparation for Cell And Tissue Cryopreservation
3. Temperature and Freezing
4. Storage in liquid nitrogen
5. Thawing/ warming Cryopreserved Cells
6. Washing and reculturing.
7. Viability Assessment/Measurement of viability.

**1. Selection of material and Cell Harvesting**

Selection of material: Selection of proper material is important.

Two important factors depend on it such as (a) nature and (b) density. Any tissue can be selected for this purpose. e.g. meristem, embryo, ovules, seeds etc.. The density should be high.

Cell Harvesting: Handle the cells gently during harvesting since damaged cells will not survive the additional damage that occurs during the freezing and thawing processes.

**What can be cryopreserved?**

1. Preservation of sperms, ova and zygote
2. Neuropreservation
3. Embryo cryopreservation
4. Ovarian Tissue Cryopreservation
5. Preservation of fungal culture

6. Preservation of microbial culture
7. Spores or mycelia from agar plate
8. Liquid mycelia
9. Preservation of bacterial culture

**2. Addition of cryoprotectant and Media preparation for Cell And Tissue Cryopreservation**

Cryoprotectants or Cryoprotective agents reduce the freezing point of the medium and also allow slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death during freezing. They are chemical which prevent cryodestruction.

Generally two cryoprotectant should be used together instead of single one as they are more effective. Examples are sucrose, alcohols, glycols, some amino acid (proline), DMSO (dimethyl sulfoxide).

Common cryoprotectants

1. Glycerol and DMSO are the most commonly employed cryoprotective agents.
2. Fetal bovine serum (FBS) is often used in mammalian cryopreservation solutions, but it is not a cryoprotective agent.
3. Salts, such as magnesium chloride, have been reported to be cryoprotective agents.
4. Dextrans, glycols, starches, sugars, and polyvinylpyrrolidone provide considerable cryoprotection in a variety of biologic systems.

**Types of cryoprotectants**

**Intracellular cryoprotectants** with low molecular weights that permeate cells. Intracellular cryoprotectants, such as glycerol and dimethyl sulfoxide at concentrations from 0.5 to 3 molar, are effective in minimizing cell damage in many slowly frozen biological systems.

**Extracellular cryoprotectants** with relatively high molecular weights that do not penetrate cells. Extracellular cryoprotective agents, such as polyvinylpyrrolidone and hydroxyethyl starch, are more effective at protecting biological systems cooled at rapid

Media for Cell and Tissue Cryopreservation: A typical media contains 90% serum + 10% cryoprotectant.

**3. Temperature and Freezing**

**Temperature:** When adding the cryopreservation media to the sample it is important that the solutions be cold (~4°C). Cell exposure to warm solutions containing DMSO can result in substantial cell damage and death.

**Freezing**: The sensitivity of cells to low temperature depends on the material.

**FREEZING METHODS FOR CRYOPRESERVATION**

There are four different types of methods:

1. **Step Down Freezing:** The samples are placed in a refrigerator overnight at 4°C, and then transferred to a -70°C (-94°F) freezer for a period of time, and moved to cryo

storage. However ,this freezing process is time consuming, difficult to repeat and document, and does not provide the controlled cooling rates and ice nucleation associated with a true controlled rate freezer. **Slow freezing method** - the tissue or plant material is slowly frozen at slow cooling rate. The advantage is the plant cells are partially dehydrated and survive better.

2. **Blast Freezing:** Blast freezing is a method designed for speed rather than maximum viability, and it's used to decrease a specific volume of material by a set temperature in a fixed amount of time. Blast freezing is commonly used for large amounts of material, like blood bags or large volumes of protein. It's important to note that there are purpose-built blast freezers designed for this process.

3. **Direct Plunge Freezing:** In Liquid Nitrogen Submersion, or Plunge Freezing, samples are loaded into a heat block, and that block is submerged into LN2 and then placed in storage. This method has been used successfully for small numbers of low volume straws and vials. **Rapid freezing method** - it involves plunging the vials in liquid nitrogen. The temperature decreases from -300 to -1000 degree rapidly.

4. **Slow Freezing using a programmable freezer:** The cell vessels/vials are placed in freezer which cools using cold nitrogen vapors. The temperature inside the cooling chamber can be accurately controlled and the time course of the temperature can be programmed. However, the time course of temperature inside the straws may be different due to the generation of heat of fusion.

5. **Dry freezing method** - in this method dehydrated cells and seeds are stored.

6. **Vitrification:** The term "Vitrification" refers to any process resulting in "glass formation", the transformation from a liquid to a solid in the absence of crystallization so the cells that are properly slow frozen become "vitrified". Vitrification involves using high concentrations of cryoprotectants to prevent ice formation but this technique is under developed.

**4. Storage**

The maintenance of the frozen cells or material at specific temperature is very important. cryopreserved cells are stored at minus 80$^{o}$C in freezer for at least 4 hours and up to 24 hours prior to transfer to an archive storage such as a freezer capable of continually maintaining temperature below minus 130°C or a gaseous phase liquid nitrogen storage vessel.In general the temperature is kept -70 to -196 degree. Prolong storage is done at temperature of -196 degree in liquid nitrogen. To prevent damage, continuous supply of nitrogen is done.

**5. Thawing**

Usually carried out by plunging the vials into warm water bath with vigorous swirling. As thawing occurs the vials are transferred to another bath at 0 degree. Thawing/Warming Cryopreserved Cells Rapid thawing (60 to 90 seconds at 37°C in water bath) provides the best recovery for most cell cultures as it reduces or prevents the formation of damaging ice

crystals within cells during rehydration. Since some cryoprotective agents may damage cells upon prolonged exposure, remove the agents as quickly and gently as possible by centrifugation

**6. Washing and reculturing**

The preserved material is washed few times to remove the cryoprotectant. This material is then recultured in a fresh medium

**7. Viability Assessment /Measurement of viability**

There is possibility of death of cells due to storage stress. Thus viability can be found at any stage.

It is calculated by formula : No of cells growing / no of cells thawed * 100

Viability Assessment :Accurate sample assessment is critical to determining preservation success and downstream utility of cell and tissue systems, for both research and clinical use. Comparing samples to pre-freeze levels 1 day after thawing with metabolic or biochemical assays provide more accurate determinations of cell viability.

**Variables to Optimize**

- Controlling the cooling rate by using an appropriate freezer
- Using appropriate cryoprotective agents
- Maintaining appropriate storage temperatures
- Controlling the warming rate

**MAJOR APPLICATION AND ADVANTAGES**

1. Once the material is successfully conserved to particular temperature it can be preserved indefinitely.
2. Reduced risk of microbial contamination /Once in storage no chance of new contamination of fungus or bacteria.
3. Minimal space required.
4. Minimal labour required.
5. It is ideal method for long term conservation of material.
6. Disease free plants can be conserved and propagated.
7. Recalcitrant seeds can be maintained for long time.
8. Endangered species can be maintained.
9. Sperms can be maintained to increase longevity.
10. Rare germplasm and other genetic manipulations can be stored.
11. Cryopreservation helps in the preservation of biological materials.
12. Cryopreservation is used to maintain the biosynthetic properties of plants.
13. Sperm, gametes, embryos, tissues, bone marrow, organ can be preserved.
14. Helps to study the adapting nature of plants and animals under the low temperature.
15. Used to preserve the genetic materials of the plants which are on the verge of extinction
16. Prevent in breeding.
17. Reduced risk of cross contamination with other cell lines

18. Reduced risk of genetic drift and morphological changes
19. **Embryo cryopreservation** is used most often to store good quality excess embryos resulting from an IVF treatment cycle.
    - Embryos can be stored for a patient who elects to have her eggs fertilized with donor sperms. Pregnancies have been reported from embryos stored for 16 years.
    - Human sperm cryopreservation is widely used to store donor and partner spermatozoa to preserve sperms.
    - It also ensure the recovery of a small number of spermatozoa in several male factor infertility.
    - It is commonly called sperm-banking is a procedure to preserve sperm cells.
20. Cryopreservation of oocyte:
    - Human oocyte cryopreservation is a new technology in which a woman's eggs are extracted, frozen or stored.
    - Egg freezing benefits two groups of women.
    - One those who are diagnosed with a medical condition
    - The second who are delaying their childbearing for personal reasons.
21. Preservation of Micro-biology cultures:
    - Bacteria and fungi can be kept short term refrigerated
    - However, cell division and metabolism is not completely arrested
    - It is not an optimal option for long term storage genetically or phenotypically as cell divisions can led to mutations.
22. In Medical Science:
    - Low temperature have been used in medicine
    - Used to prevent food spoilage
    - Now- a- days it is used in fertility treatment the transport of human organs and the long- term storage of biological specimens
    - To conserve plant biodiversity
23. In Animal Husbandry:

    The introduction of cryopreservation technology leads a major breakthrough in animal husbandry. Since the 1st successful cryopreservation of bull semen has been used to propagate the rare and endangered species using assisted reproduction techniques. Every year, more than 25 millions cows are artificially inseminated with frozen-thawed bull semen8 and many bovine calves have been produced using the transfer of cryopreseved embryos into cow

**DISADVANTAGES**

There are few disadvantages to storing eggs.

- During the cycle where the eggs are harvested, patients undergo a traditional IVF protocol.
- There are known side effects with fertility medication including the risk of ovarian hyper stimulation syndrome or OHSS.

- The lengthy process of slow-rate freezing and the subsequent long-term storage of these valuable cells can often be costly, consuming large amounts of energy to accurately maintain such low temperatures.

Ovarian hyper stimulation syndrome (OHSS) :

- Ovarian hyper stimulation syndrome (OHSS) affects women taking injectable hormone medications to stimulate the development of eggs in the ovaries.
- This may occur in women undergoing in vitro fertilization (IVF), ovulation induction or intrauterine insemination.
- Too much hormone medication in system can lead to OHSS, in which ovaries become swollen and painful.
- A small number of women may develop severe OHSS, which can cause rapid weight gain, abdominal pain, vomiting and shortness of breath.
- Less often, OHSS happens during fertility treatments using medications you take by mouth, such as clomiphene (Clomid, Serophene).

**OBSTACLES IN CRYOPRESERVATION**

1. Upto 60% human body is composed of water: Actually the freezing point of water is 0 degree centigrade while the cryoscopy temperature can be as low as -90 degree centigrade.
2. Very expensive Technique
3. Ice formation can result in the needle shaped crystals resulting in the damage to cell membrane.
4. Unequal distribution or over distribution of cryoprotectants.
5. Moreover, thermal gradients can induce mechanical stress due to uneven expansion or contraction in the biomaterial.
6. The cooling rate required for optimal survival varies by several orders of magnitude between different cell types.
7. Mass transfer limitations