CRYOPRESERVATION

UNIT 1: GENERAL LABORATORY TECHNIQUES

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INTRODUCTION

Cryopreservation: It is a process where organelles, cells, tissues, extracellular matrix, organs, or any other biological constructs susceptible to damage caused by unregulated chemical kinetics are preserved by cooling to very low temperatures (typically −80 °C using solid carbon dioxide or −196 °C using liquid nitrogen)

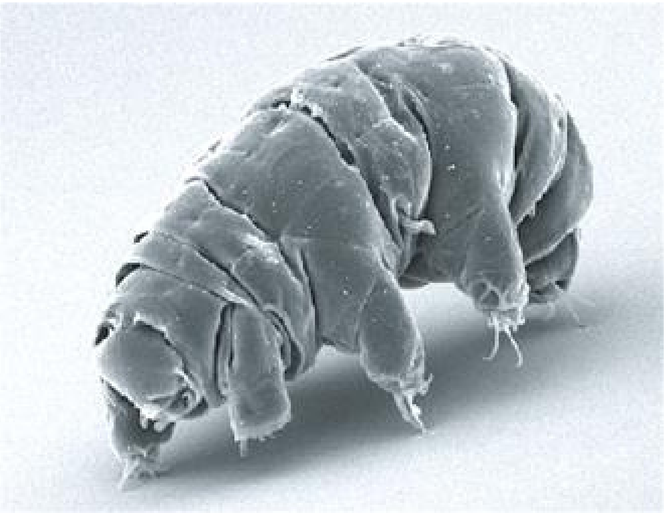
It is based on the reduction and subsequent arrest of metabolic functions of biological material by imposition of ultra low temperature.

Cell viability is adversely effected.

To prevent this, traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed ***cryoprotectants***. New methods are being investigated due to the inherent toxicity of many cryoprotectants.

Natural cryopreservation

Water-bears (Tardigrada), microscopic multicellular organisms, can survive freezing by replacing most of their internal water with the sugar ***trehalose***, preventing it from crystallization that otherwise damages cell membranes.



Tubes of biological samples being placed in liquid nitrogen.

- Cryogenically preserved samples being removed from a liquid nitrogen*dewar.*

Cryogenic storage dewar

A **cryogenic storage dewar**(named after James Dewar) is a specialised type of vacuum flask used for storing cryogens (such as liquid nitrogen or liquid helium), whose boiling points are much lower than room temperature.

Cryogenic storage dewarsmay take several different forms including open buckets, flasks with loose-fitting stoppers and self-pressurisingtanks.

All dewarshave walls constructed from two or more layers, with a high vacuum maintained between the layers. This provides very good thermal insulation between the interior and exterior of the dewar, which reduces the rate at which the contents boil away.

A self-pressurisingdewar(silver, foreground) being filled with liquid nitrogen from a large storage tank (white, background).

Principle

Cryopreservation is based on the ability of certain small molecules to enter cells and prevent dehydration and formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the freezing process.

Techniques

1. **Collection & preservation of cells and tissues**
2. **Addition of Cryoprotectants**
3. **Freezing/Cooling**
   - Slow freezing method
   - Rapid freezing method
   - Vitrification
4. **Storage**
5. Thawing or Warming (Bringing them back to room temperature)

Step 1. Collection of Cells

What can be cryopreserved?

Generally, cryopreservation is easier for thin samples and suspended cells, because these can be cooled more quickly and so require lesser doses of toxic cryoprotectants. Therefore, cryopreservation of human livers and hearts for storage and transplant is still impractical.

Semen in semen cryopreservation

Bl ood

Stem cells. It is optimal in high concentration of synthetic serum, stepwise equilibration and slow
Special cells for transfusion like platelets (Thrombosomesby Cellphire)

cooling.

Umbilical cord blood in a Cord blood bank

Tissue samples like tumors and histological cross sections

Eggs (oocytes) in oocyte cryopreservation

Step 2. Addition of Cryoprotectants

Cryoprotectant:A substance used to protect biological tissue from freezing damage (i.e. due to ice formation).

They are characterized as:

1. ***Permeating:***DMSO, Methanol, Glycerol
2. ***Non-permeating:***Sugars (trehalose), sugar alcohols, high MW polymers, PVP, hydroxyl ethyl starch

Glycerol is used primarily for cryoprotection of red blood cells, and ***DMSO***is used for protection of most other cells and tissues. A sugar called ***trehalose***, which occurs in organisms capable of surviving extreme dehydration, is used for freeze-drying methods of cryopreservation. Trehalose stabilizes cell membranes, and it is particularly useful for the preservation of sperm, stem cells, and blood cells.

Addition of osmotically active compounds like mannitol, sorbitol, sucrose and proline increase the freezing resistance of cells. They reduce the mean cell volume and increase their post freezing survival.

A combination of cryoprotectants can be more effective.

Step 3. Freezing/Cooling

Freezing cells includes intracellular and extracellular complex events that are not fully understood.

During the freezing process ice forms in and out the cell and this is very much dependent on the cooling rate. If ice forms externally to the cell, water migrates out of the cell causing dehydration, shrinkage and finally cell death. If too much water remains inside the cell during the freezing process, intracellular ice crystals form that damage cellular organelles and pierce the cell membranes during the thawing process.

Rapid cooling minimizes the solute imbalance between the internal and external of the cell, but more intracellular ice is formed. In Slow cooling intracellular water migrates out of the cell resulting dehydration and shrinkage (Elliot, Wang, & Fuller, 2017).

Cells may be successfully frozen when the cooling rate is slow enough to prevent intracellular ice formation but fast enough to prevent dehydration and damage.

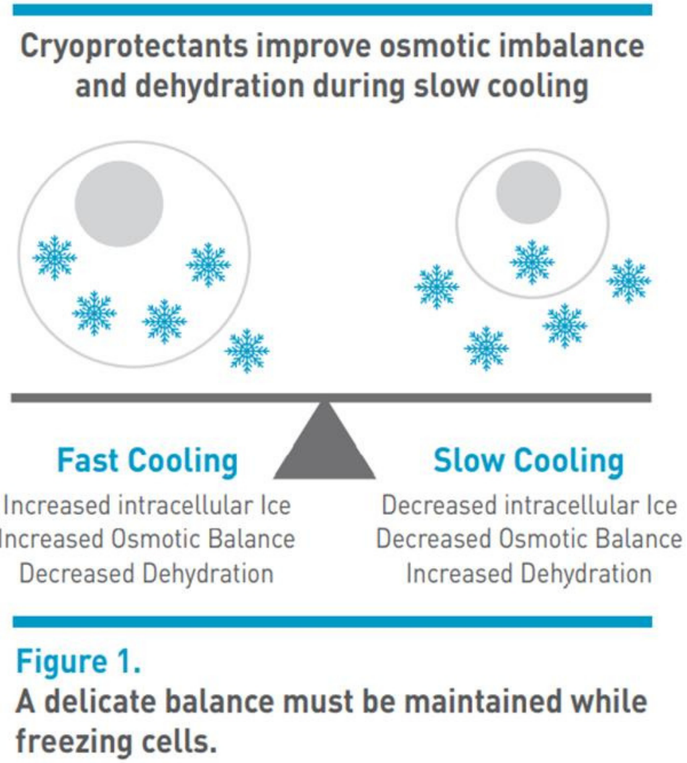


Figure 1.
A delicate balance must be maintained while freezing cells.

Risks

Phenomena which can cause damage to cells during cryopreservation mainly occur during the freezing stage, and include:

Solution effects: As ice crystals grow in freezing water, solutes are excluded, causing them to become concentrated in the remaining liquid water. High concentrations of some solutes can be very damaging.

Extracellular ice formation: When tissues are cooled slowly, water migrates out of cells and ice forms in the extracellular space. Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing.

Dehydration: Migration of water, causing extracellular ice formation, can also cause cellular dehydration. The associated stresses on the cell can cause damage directly.

Intracellular ice formation: While some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells.

Freezing methods:

Slow Freezing: Used traditionally, 60-70% survival rate

Rapid Freezing: Midway technique between slow freezing and vitrification

Vitrification or Ultra-rapid Cooling: Latest technology, 99-100 % survival rate

Slow Freezing: Manual freezing

Decreasing the temperature of the semen while adding a CPA in a stepwise manner and after plunging into liquid nitrogen.

Cooling rate of the specimen from room temperature to 5 degree Celsius is 0.5-1 degree Celsius/min.

The sample is then frozen from 5 degree Celsius to -80 degree Celsius at a rate of 1-10 degree Celsius/min. The specimen is then plunged into liquid nitrogen at -196 degree Celsius.

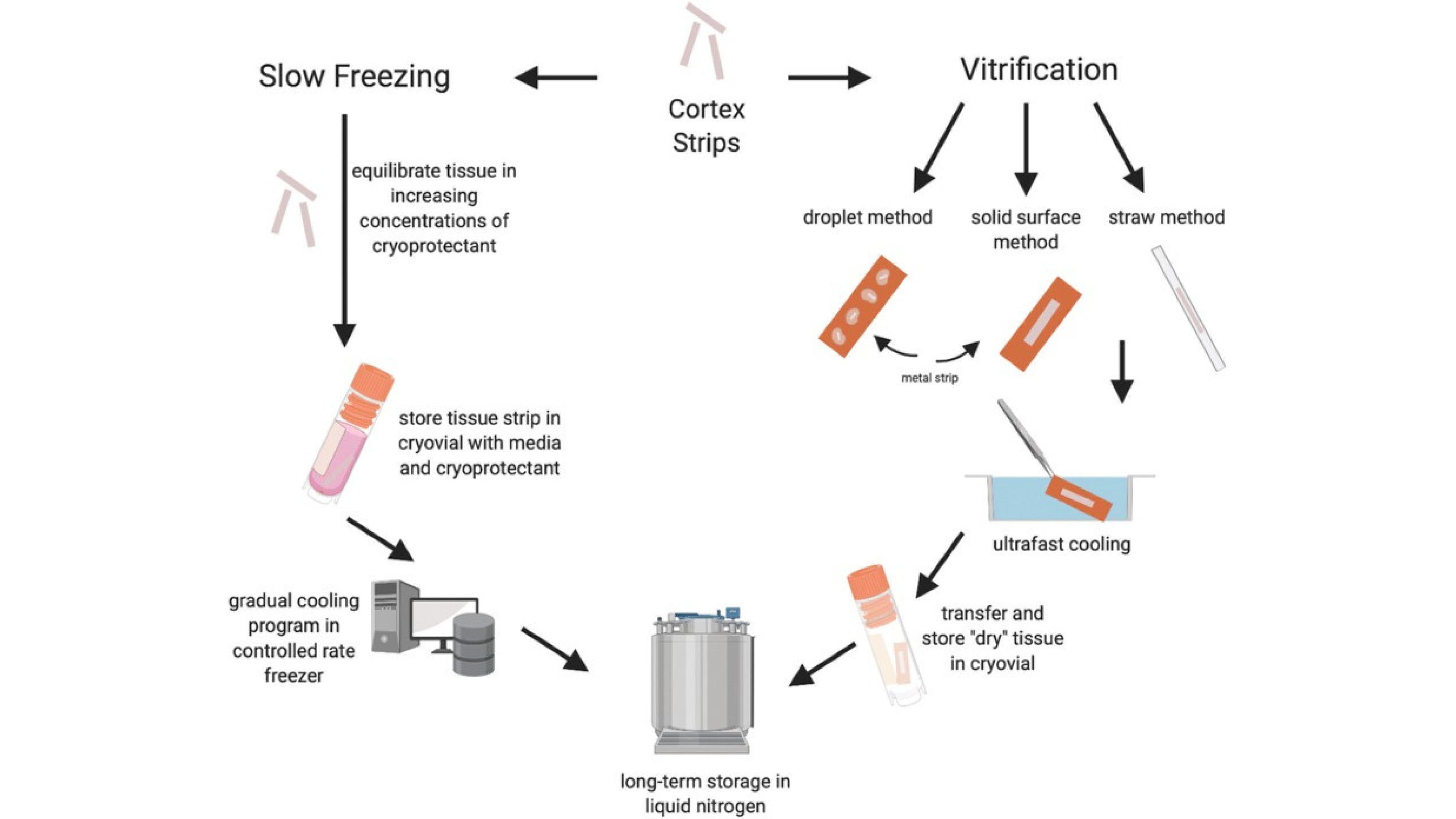
Slow programmable freezing

The slow freezing protocol includes a pre-equilibration step of oocytes or embryos in low concentrations of permeable cryoprotective agents (CPA ~10% v/v) before the cooling step.

The embryos are then taken from room temperature, placed in special straws, sealed, to approximately −90 °C (−130 °F) in a machine with an integrated computer where embryos were cooled down step-wise (max. 0.5 ◦ C/min) in a programmable system, after artificial extracellular ice seeding is induced.

During cooling, for each formation of an extracellular ice crystal, the cell will re-establish osmotic equilibrium by dehydration. Thereby, during cooling, cells continue to dehydrate intracellularly, reducing the risk for intracellular ice crystal formation until the straws are plunged into LN2.

It emerges from the preceding studies that the common denominator that negatively affects post-thaw survival is uncontrolled ice crystal formation inside and outside the cell. This is the crucial factor directly affecting the success of the slow freezing technique.


Slow Freezing
Cortex Strips
Vitrification
equilibrate tissue in increasing concentrations of cryoprotectant
droplet method
solid surface method
straw method
metal strip
store tissue strip in cryovial with media and cryoprotectant
ultrafast cooling
gradual cooling program in controlled rate freezer
transfer and store "dry" tissue in cryovial
long-term storage in liquid nitrogen

Rapid Freezing

It is a midway technique between slow freezing and vitrification.

It is quicker than slow-freezing technique, does not involve the use of programmable machines and requires lower concentrations of cryoprotectant agents (CPA) than those used in vitrification.

Experimental results demonstrate that this technique has lower performances than slow freezing’s and vitrification’s one

Rapid freezing technique is applied for the cryopreservation of shoot tips and somatic embryos.

Vitrification

Almost 80 years ago, Luyethighlighted that the intracellular control of the foci of nucleation and ice crystal formation is a crucial event that determines the viability of all cell types that go through cryopreservation.

He stressed that the change in the state of aggregation from liquid water into ice crystals in the intracellular compartment must be considered principally as the first cause of cell death.

To counteract the crystallization process, Luyetintroduced an alternative concept called vitrification.

The general principle of vitrification is to convert a liquid into a glass-like amorphous solid that is free of any crystalline structures.

At Which Temperature Do We Obtain a Glass-Like Amorphous Solid State?

Solidification of pure water in a glassy solid form (vitrification) is achieved when the temperature decreases extremely rapidly below the glass transition temperature (Tg).

Tgfor pure water is -137 degree Celsius and only possible with rapid cooling rates (15000–30000°Cmin−1) to avoid spontaneous crystal nucleation when crossing the zone between equilibrium melting temperature (Tm) and Tg.

Below Tg, the movement of water molecules is too slow to organize the start of crystallization, and the solution solidifies with water molecules arranged in a completely disordered state.

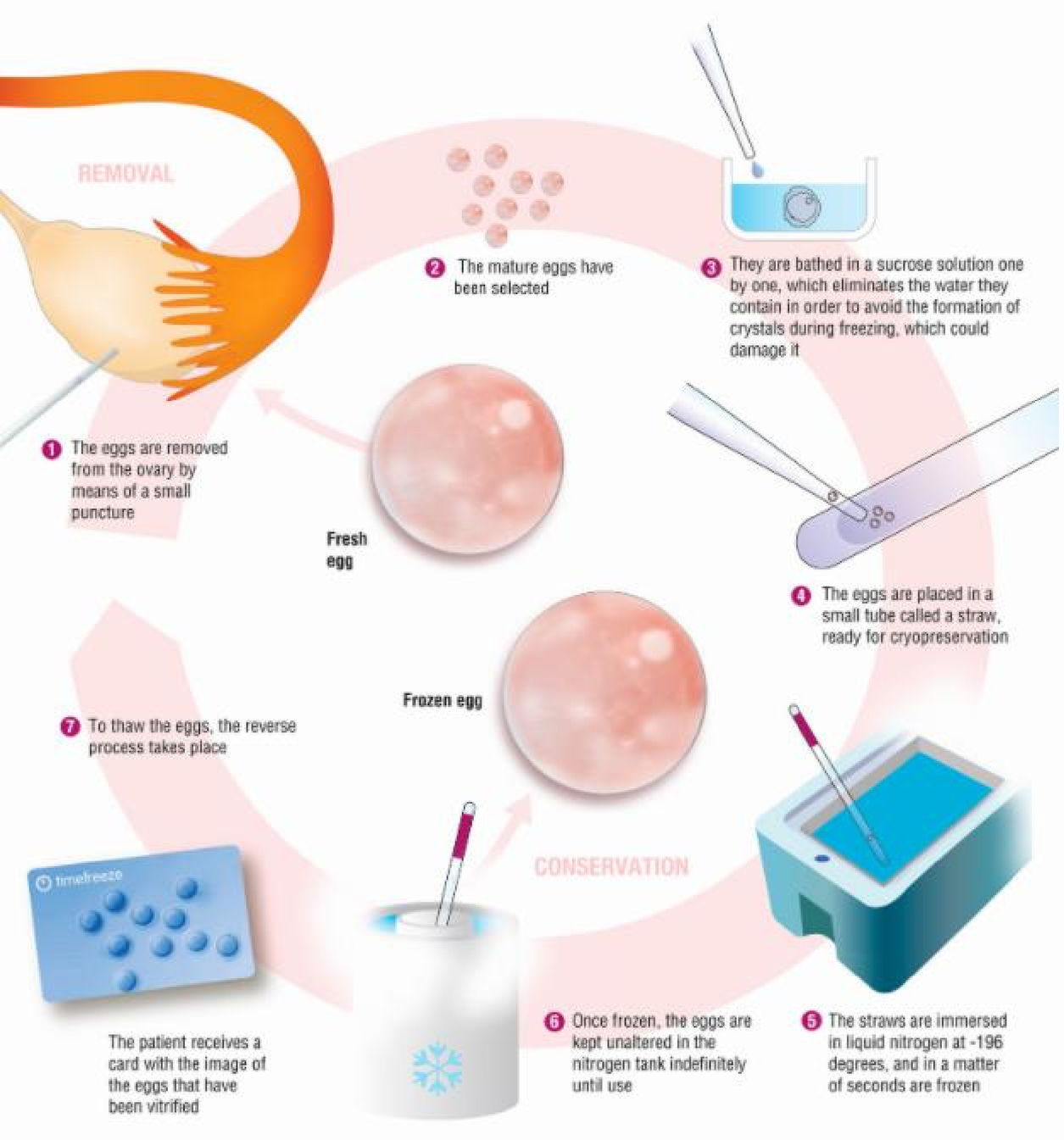
important.

Low temperatures in association with extreme increase in viscosity are

CPAs increase the viscosity and thereby lead to slowdown of the molecular movements of water.

An increase in viscosity results in: (i) a delay of nucleation phenomenon, (ii) a reduction of growth rate of ice crystals, (iii) a limitation in the size of crystals between Tmand Tg, (iv) increase in Tg.

Tg= −135 ◦C for pure water, < −135 ◦C for CPA solutions

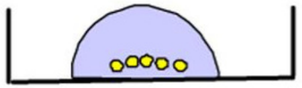


- Vitrification is now widely used for cryopreservation of unfertilized embryos and eggs.

All procedures were done at room temperature

1. Vitrification

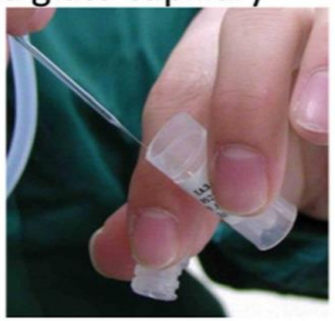
Embryos were immersed in equilibrium solution for 3min.



3 min

Equilibrium solution
5%DMSO+5%EG-PB1 50μl

Transfer embryos into a tube containing vitrification solution with a glass capillary

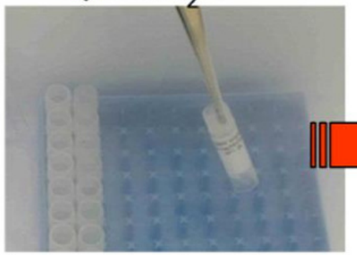


Vitrification solution
EFS42.5c-d 50μl

1 min

Centrifuge at 280 x *g* for about 1 sec, if necessary

Directly immerse in LN_2

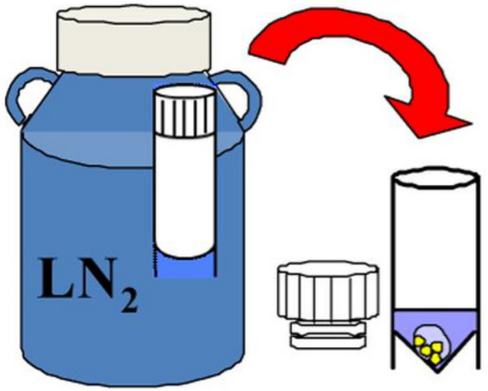


LN_2

preservation in LN_2

2. Liquefy (thawing & recovery)

Retrieve a tube and let it stand for 3min.



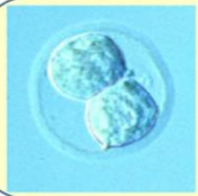
Add 850 μl of TS-1 (thawing solution, 0.5M sucrose-PB1)

3 min

3 min

Transfer the entire volume to a dish with 1ml tip

Transfer embryos and wash in 50 μl of TS-2 (0.25M sucrose-PB1)



After washing, wait for 0-3 min the shape of embryo become to shrink like a picture.

Culture in medium

Challenges to Vitrification:

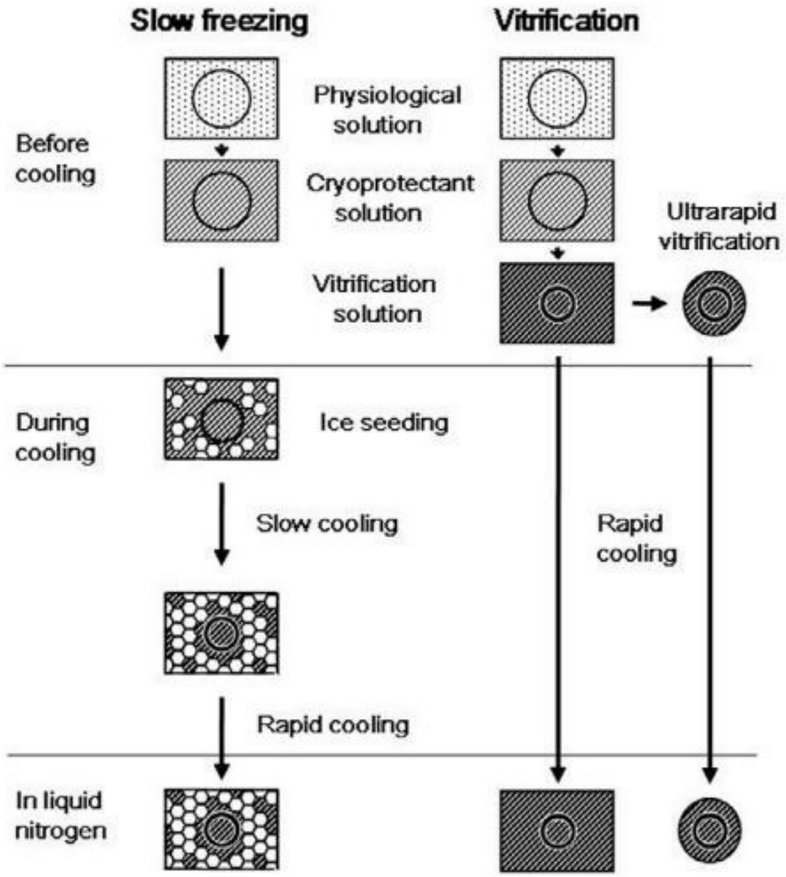
For established methods of cryopreservation, the solute must penetrate the cell membrane in order to achieve increased viscosity and decrease freezing temperature inside the cell. Sugars do not readily permeate through the membrane. Those solutes that do, such as ***dimethyl sulfoxide***, a common cryoprotectant, are often toxic in intense concentration.

One of the difficult compromises of vitrifying cryopreservation concerns limiting the damage produced by the cryoprotectant itself due to cryoprotectant toxicity.

Mixtures of cryoprotectants and the use of ice blockers have enabled the successful vitrification of a ***rabbit kidney*** to −135 °C with their proprietary vitrification mixture. Upon rewarming, the kidney was transplanted successfully into a rabbit, with complete functionality and viability,

Twenty-First Century Medicine

Slow freezing Vs Vitrification



Schematic presentation of an embryo (circle) before cooling, during cooling and in liquid nitrogen in slow freezing, conventional straw vitrification, and ultrarapid vitrification. White hexagons represent ice crystals.

Characteristics	Vitrification	Slow-freezing
Direct contact with liquid nitrogen	Yes	No
Ice formation	No	Yes
Time	Fast (minutes)[a]	Slow (hours)
CPA equilibration	Yes	Yes
CPA concentration	High (over 40%)	Low (10–15%)
Sample size (human)	Up to 5 × 1 × 1 mm[b]	Up to 2 × 4 × 12 mm[c]
Cooling rates (°C/min)	15,000–30,000	0.15–0.30
Cost	Protocol-dependent (usually inexpensive)	Equipment-dependent (usually expensive)
Special equipment	No	Yes
Technical expertise	Risky	Simple
Routinely applied for cryopreservation of human ovarian tissue	No	Yes

Adapted from Moore and Bonilla (2006).
[a]Considering just one cryocycle.[b]Li et al. (2007) and Huang et al. (2008).[c]Donnez et al. (2004).

Step 4. Storage

The frozen cultures should be maintained at the specific temperature.

Generally, the frozen cells/tissues are maintained at temperatures in the range of -70 to -196°C for storage.

Although, with temperatures above -130°C, ice crystal growth may take place inside the cells which decreases viability of cells.

The ideal storage is done in liquid N2 refrigerator at 150°C in the vapourphase, or at -196°C in the liquid phase.

The final aim of storage is to halt all the cellular metabolic activities and preserve their viability.

The temperature at -196°C in liquid nitrogen is regarded as ideal for long term storage.

A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is necessary.

It is essential to check the viability of the germplasm time and again in some samples.

Proper documentation of the germplasm storage should be done.

Step 5. Thawing

Thawing is usually performed by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with robust swirling.

By this process, rapid thawing (at the rate of 500-750°C min-1) takes place, and this preserves the cells from the damaging effects from ice crystal formati on.

As soon as the thawing occurs (ice completely melts), the ampoules are transferred to a water bath at temperature 20-25°C at the same instant.

The cells get damaged if left in warm (37-45°C) water bath for long time.

For the cryopreserved material (cells/tissues) where the water content has been decreased to an optimal level before freezing, the process of thawing becomes less vital.

Step 6. Recovery of Cryopreserved Cells

1. Prepare a culture vessel (T-75 flask) so that it contains at least 10 mL of the appropriate culture medium equilibrated for temperature and pH.
2. Remove the vial from the liquid nitrogen freezer and thaw by gentle agitation in a 37°C water bath (or a bath set at the normal growth temperature for that cell line). Thaw rapidly until ice crystals have melted (approximately 2 minutes).
3. Remove the vial from the water bath and decontaminate it by dipping in or spraying with 70% ethanol. Follow strict aseptic conditions in a laminar flow tissue culture hood for all further manipulations.
4. Unscrew the top of the vial and transfer the contents to a sterile centrifuge tube containing 9 mL complete growth medium. Remove the cryoprotectant agent by gentle centrifugation (10 minutes at 125 ×g). Discard the supernatant, taking care not to disturb the soft pellet, and resuspend the cells in 1 mL or 2 mL of complete growth medium. Pipette gently to loosen the pellet and break apart clumps. (If the cells normally grow as clusters, avoid over-pipetting during resuspension.) Transfer the cell suspension into the medium in the culture vessel and mix thoroughly.
5. Examine the cultures after 24 hours and subculture as needed.

Applications of Cryopreservation

The applications of cryopreservation can be categorized into the following areas:

1. Cryopreservation of cells or organs;
2. Cryosurgery: Cryosurgical techniques are less invasive and have lower morbidity compared with surgical resection. However, the use of cryosurgery has been limited by a lack of good understanding of the underlying mechanisms of tissue destruction. To apply cryosurgery clinically, and to extend its use, it is important to understand the mechanisms of freeze injury on cells, and to control the thermal parameters.
3. Biochemistry and molecular biology;
4. Food sciences;
5. Ecology and plant physiology; and
6. Many medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and in vitro fertilization (IVF)

Medical Science & Food Sciences

Low temperature have been used in medicine and to prevent food spoilage since ancient time. Now-a-days it is used in fertility treatment the transport of human organs and the long-term storage of biological specimens, either for future or simply as a record of biodiversity.

Cryopreservation of sperm:-Today human sperm cryopreservation is widely used to store donor and partner spermatozoa before assisted reproduction treatments to preserve spermatozoa before therapy for malignant diseases, vasectomy or surgical in fertility treatments and to ensure the recovery of a small number of spermatozoa in several male factor infertility. It is commonly called sperm-banking which is a procedure to preserve sperm cells. For human sperm the longest successful storage is 21 years.

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 whereby the necessary treatments for cure may render them sterile or unable to produce viable eggs. The second who are delaying their childbearing for personal reasons. Eggs frozen at the age of 35 are more usable than fresh oocytes produced at age 43 years of age.

Cryopreservation of testicular tissue:-Cryopreservation of immature testicular tissue is a developing method to avail reproduction to young boys who need to have gonadal toxic therapy.

Embryo cryopreservation:-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.

Cryopreservation of ovarian tissue :-Ovarian tissue cryopreservation is considered to be an experimental technique for fertility preservation. This procedure is an option for patients who require immediate gonadotoxic treatment of aggressive malignancies when there is insufficient time to allow the woman to undergo ovulation induction, oocyte retrieval and cryopreservation oocytes and/or embryos. Ovarian tissue cryopreservation is the only option available for fertility preservation in young girls who are prepubertal or in woman who have hormone-sensitive malignancies or whose reproductive potential is threatened by future of cryopreservation.

Cryopreservation of stem cell:-An important application of cryopreservation is in the freezing and storage of hematopoietic stem cell, which are found in the bone marrow rescue, hematopoietic stem cells are collected from a patient's bone marrow prior to treatment with high-dose chemotherapy. Following treatment, the patient's cryopreserved cells are thawed and infuse back into the body. This procedure is necessary, since high dose chemotherapy is extremely toxic to the bone marrow

Preservation of micro-biology cultures

Bacteria and fungi can be kept short term refrigerated however, cell division and metabolism is not completely arrested and thus is not an optimal option for long term storage or to preserve cultures genetically or phenotypically as cell divisions can led to mutations.

To conserve plant biodiversity

For long-term conservation cryopreservation is the most effective tool, as it maintain the living cells, tissues, organs at ultralow temperature(usually that of liquid nitrogen,-196ºc).At liquid nitrogen temperature, all metabolic activity and cell divisions are stopped and cells will not undergo genetic changes during storage. Cryopreservation is the one technique that ensures the safe an cost-efficient long term conservation of various categories of plants, including non-orthodox seed species, vegetatively propagated plants, rare and endangered species and biotechnology products.

Future of Cryopreservation

Vitrification method of cryopreservation may bring new opportunities to research protocols. It is still an experimental procedure. There are two major concern about vitrification -toxicity of high concentration of cryoprotectants used and microbial contamination of liquid nitrogen.

Several IVF programs have adopted the vitrification method as the sole procedure for day-3 human embryos and for human blastocysts, with excellent survival and pregnancy rates.

The challenge now is to find a protocol to successfully vitrify human oocytes for which the slow freezing method has yet to produce acceptable.

The practical application of cryopreservation in the aquatic species needs more vigorous research efforts in this area and the efforts may be prioritized on endangered, economical value and representative species from various aquatic habitats.

The establishment of cryobanksto utilize the cryopreservation world-wide would be a significant and promising task in future.