ADVANCES IN CRYOPRESERVATION, CRYOPROTECTIVE AGENTS AND CRYOPRESERVATION OF CELLS LINES - A BOON IN IVF TECHNOLOGY.

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ABSTRACT *To ensure reproducible results and continuity in research and biomedical processes, today's scientists are faced with the task of genetically stabilizing living cells. Serial sub culturing is time consuming and can lead to contamination or genetic drift as smaller and smaller portions of a population are selected. However, a population of cells can be stabilized by subjecting them to cryogenic temperatures which; for all practical purposes, stops time. Stabilizing cells at cryogenic temperatures is called cryopreservation, an applied aspect of cryobiology, or the study of life at low temperatures. Advances in cryopreservation technology have led to methods that allow low-temperature maintenance of a variety of cell types. Techniques are available for the preservation of microorganisms, isolated tissue cells, small multicellular organisms, and even more complex organisms such as embryos.*

Keywords: *Cryogenic, cryobiology, cryopreservation*

Introduction:

The freezing process involves complex phenomena that, even after decades of research, are not fully understood. Cryobiological studies have led to speculation on what occurs during the freezing of living cells and how adverse phenomena can be overcome. Since water is the major component of all living cells and must be available for the chemical processes of life to occur, cellular metabolism stops when all water in the system is converted to ice.

Ice forms at different rates during the cooling process. During slow cooling, freezing occurs external to the cell before intracellular ice begins to form. As ice forms, water is removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as intracellularly, can be detrimental to cell survival. If too much water remains inside the cell, damage due to ice crystal formation and recrystallisation during warming can occur. The rate of cooling has a dramatic effect on these phenomena.

Rapid cooling minimizes the solute concentration effects as ice forms uniformly, but leads to more intracellular ice. Slow cooling, on the other hand, results in a greater loss of water from the cell and less internal ice, but increases the solution effects. Cell permeability affects the rate of water loss; more permeable cells are able to tolerate rapid cooling better than less permeable cells. Mazur et al. al. have postulated that ice crystal formation and solution effects both play a role in cell inactivation, and that an optimum cooling rate minimizes the effect of each. With few exceptions, a cooling rate of 1°C per minute is preferred. Using cryoprotective additives or chemicals that protect the cells during freezing can also minimize the detrimental effects of increased solute concentration and ice crystal formation. The most commonly used cryoprotective agents are dimethylsulfoxide (DMSO) and glycerol. Additionally, maintaining frozen cells at the proper storage temperature and using an appropriate warming rate will minimize damage to frozen cells.

Seed Lot System:

To ensure the genetic stability of a culture, the number of passages from the original must be minimized. When freezing cells use a system that ensures that early passage material is always available for producing new working stock. One method of preserving early passage material is to use a seed lot system.

When preparing the first frozen lot of a culture, a portion of the lot is set aside as seed material. The vials designated as seed material are maintained separately from the working stocks to ensure that they remain unused and are not handled during retrieval operations. When the first working stock lot is depleted, a vial is retrieved from the seed lot and used to prepare a second working stock. This continues until all seed vials, except one have been depleted. The last seed vial is then used to prepare a second seed lot. The second seed lot remains only one or two passages from the original material, but may be separated by many years if the lots are adequately sized.

In addition to seed material, a small portion of the original lot should be segregated and maintained in a location remote from all other material. Reserve stocks ensure that strains are not lost in the event of a physical disaster at the primary location. Using seed lots and maintaining off-site reserve material are of primary importance in ensuring continuity and longevity in any well-managed culture collection.

Cryoprotective Agents:

Many compounds have been tested as cryoprotective agents, either alone or in combination, including sugars, serum and solvents. Although there are no absolute rules in cryopreservation, glycerol and DMSO have been widely used and seem to be most effective.

Cryoprotective agents serve several functions during the freezing process. Freezing point depression is observed when DMSO is used which serves to encourage greater dehydration of the cells prior to intracellular freezing. Cryoprotective agents also seem to be most effective when they can penetrate the cell and delay intracellular freezing and minimize the solution effects. The choice of a cryoprotective agent is dependent upon the type of cell to be preserved. For most cells, glycerol is the agent of choice because it is usually less toxic than DMSO. However, DMSO is more penetrating and is usually the agent of choice for larger, more complex cells such as protists.

The cryoprotective agent should be diluted to the desired concentration in fresh growth medium prior to adding it to the cell suspension. This minimizes the potentially deleterious effects of chemical reactions such as generation of heat, and assures a more uniform exposure to the cryoprotective agent when it is added to the cell suspension, reducing potential toxic effects. DMSO and glycerol are generally used in concentrations ranging from 5 to 10% (v/v), and are not used together in the same suspension with the exception of plant cells. The optimum concentration varies with the cell type and the highest concentration the cells can tolerate should be used. The Quick-Reference Chart lists the recommended concentrations of cryoprotective agents for each group of cells and serves as a general guide to choosing the proper agent.

Glycerol may be sterilized prior to use by autoclaving for 15 minutes at 121 °C and 15 lb. Glycerol should be protected from light during storage. DMSO must be sterilized by filtration using a 0.2 t nylon syringe filter or a Teflon syringe filter which has been pre-washed with alcohol and rinsed with DMSO. Cryoprotective agents should be prepared in single use volumes to minimize the risk of contamination and moisture introduction with repeated use from one container.

Caution must be observed when handling DMSO as it is quickly absorbed into the body through the skin and may transport harmful substances into the body with it.

Preparation of Cells:

Several factors must be considered when preparing cells for cryopreservation. These include the type of cell, cell viability, growth conditions, and physiological state of the cells, the number of cells, and how the cells are handled. When preparing the initial seed stock of a new isolate or cell line, the culture should be examined for identity and contaminating microorganisms. This examination should be repeated after preservation and each time a new lot of the culture is prepared.

Cells in culture undergo continuous cell division. Several factors come into play that affects the properties of the cells. Freezing cells attempts to stabilize changes in cells. Reasons for freezing cells:

- Genotypic drift due to genetic instability
- Senescence
- Transformation
- Phenotypic instability (selection and dedifferentiation)
- Contamination
- Cross contamination with other eel! Lines
- Incubator failure
- Save time and material
- Distribution to other users
- Cryobiology - study of life at low temperature
- Looking to increase the Genetic Stability of Living Cells Cryopreservation stops time

Various techniques available to preserve microorganisms, isolated tissue cells, small multicellular organisms and embryos. Water in the cells is slowly converted to ice. Water is important for cellular metabolism. Thus cell metabolism stops at cryopreservation.

Harvesting Cells for Freezing:

Avoid damaging cells during harvest. Remove all dissociating agents' thorough washing and inactivation of enzymes. Centrifuge cells at low speed for 10 min pool the contents of all harvested flasks ensure uniformity of sample. QC testing is easier. Use 106 to 10 cells / ml.

Use preservative agent, slow cooling allows water to move out before freezing, store at - 135° (retards growth of ice crystals),rapid warming of cells between _500 and 00, this range is considered to be when most damage occurs.

Cryopreservative agents are used alone or in combinations like sugars, serum. Solvents. Most common compounds are DMSO and Glycerol. They lower the freezing point, low toxicity, penetrate membranes freely, strongly bind water to prevent it freezing at 0, slow cooling rate allows water to leave cell and prevents membrane damage. These should be reagent grade, filter sterilized.

Microorganisms:

Microbial cells, particularly bacteria and yeast, grown under aerated conditions demonstrate a greater resistance to the detrimental effects of cooling and freezing than non-aerated cells. T. Nei *et. Al,* have demonstrated that cell permeability is greater in aerated cultures, and that the aerated cells dehydrate faster during cooling than non-aerated cells.

Microbial cells harvested from late log or early stationary cultures also demonstrate greater resistance to the freezing process than younger or older cells. These can be conveniently harvested from agar slants or plates, or when greater quantities are required, grown in broth culture and harvested by centrifugation. In either case. Cells are generally suspended in fresh growth medium containing the cryoprotective agent.

Genetically modified organisms can be preserved in a manner similar to the unmodified host cell in most cases. The viability and an estimate of recovery should be determined both before and after freezing the culture. Viability is a measure of the culture's ability to grow and reproduce. A comparison of the counts prior to and after freezing gives an indication of the degree of recovery or the success of the preservation procedure.

Animal Cell Lines:

When preparing animal cells for cryopreservation. cell populations need to be adjusted to levels that ensure adequate recovery without unnecessarily growing large numbers of cells. For most mammalian cells, a starting population between 106 to 107 cells/ml is optimum. Alternatively, the cell pellet can be resuspended in the cryoprotectant (1 x cryoprotective agent + medium) to the desired cell concentration. Gentle handling during cell harvesting and concentration procedures will ensure healthy cells prior to subjecting them to cold stress. Where appropriate, the pH should be maintained by gassing with 5% or 10% CO_2. Animal cell cultures are especially susceptible to contamination by other cells, such as HeLa cells, and contaminating microorganisms. The species of origin of cell lines should be verified by isoenzyme analysis, karyotyping, and/or immunological assays. These should be performed prior to and following preservation. Contamination by viruses and Mycoplasma sp. is of particular concern. A good characterization program for animal cell lines should include a check for contamination by bacteria, fungi, appropriate viruses, mycoplasma, and in some cases, protozoa.

Cell Line Characterization:

Cell lines are important to keep records for future use. Use of isolated cells are not always clear at the first time but may develop into very useful cell line, especially important if cells become widely used, particularly if cells used in product requiring government validation or detail origin and handling of original cells.

Requirement for Cell Line Characterization:

Cell line characterization is must to confirm species of origin. Tissue of origin can be characterized by chromosomal analysis. To identify the lineage we can use certain enzymes or surface markers. Position within lineage (stem cell, precursor, differentiation) should also be identified for a cell. Cell lineage can be identified by cell surface antigens, intermediate filament proteins, differentiated products / functions, lineage specific enzymes or unique markers like MHC, HLA, or by DNA fingerprinting.

We must identify the status of transformation, verify absence of cross contamination and determine if cells are prone to genetic instability. Study of morphology of a cell is difficult as cells appear differently under culture conditions but can be studied using staining, microscopy, and photography.

Chromosome Analysis:

The chromosomal analysis is used to identify cell lines regarding species and sex of original tissues. We can distinguish normal cells from malignant tissues through the chromosomal analysis. The chromosomes are stained by several stains and they give banded appearance. After the staining, it is so easy to count the number of chromosomes when these are visible in mitosis.

Karyotype:

Karyotype is the representation of the chromosome set in an individual or species described in terms of both the number and structure of the chromosomes. This term is used to the group of characteristic that identifies a particular chromosome set and is usually represented by a diagram called idiogram, where chromosome of haploid set of an organism are ordered in a series of decreasing size. Karyotype also suggests primitive or advanced feature of an organism. Karyotypes in which linear differentiation due to distinct banding patterns allow identification of chromosomes that are similar in morphology. The banding technique allows detection of GC or AT rich regions or the regions with repetitive DNA. There are some other options like chromosome painting, using of specific DNA probes, hybridization with fluorescent labeled probe and ID visually or via southern blotting.

In *vitro* Transformation of Animal Cells:

Transformation is the series of events that causes genetic instability that changes cell line properties including growth rate, mode of growth (loss of contact inhibition), specialized product formation, longevity, and loss of need for adhesion Transformation vs. Transfection.

Introduction of DNA into a Cell (like Viral DNA):

Microbial Transformation—changes in phenotype due to uptake of genetic material.

Transfection—used for same process in mammalian cells. They may lead to gene expression, activation of cellular or viral gene, produce mRNA by transcription, or protein product by translation. It may lead to transformation of exogenous DNA into cultured cells, permanent, heritable change in phenotype, may be caused viral infection or transfection, or may be caused spontaneously (ionizing or carcinogens).
Transformation results in genetic instability

- Immortalizations
- Aberrant Growth Control
- Malignancy
- immortalization can happen alone, the others tend to occur together.

Transfection Methods:

Chemical Transfection Methods produce a chemical environment that causes DNA to attach to cells.

1. **Calcium phosphate transfection**

- Calcium phosphate (CaPO4) commonly used
- DNA, Car, and phosphate mixed
- Form precipitate which is taken up by cell
- CaPO4 and DNA precipitate and form sediment
- Sediment adsorbs to cell
- DNA taken up by Ca+ requiring process
- Most likely via endocytosis
- DNA is either transiently expressed or integrated into host chromosome
- inexpensive and works on bacterial and mammalian cells

2. **DEAE-dextran transfection** is also commonly used. It is more reproducible than CaPO4 and may give higher efficiency.
3. **Liposome transfection:** They are positively charged lipids. They spontaneously interact with DNA—form complex and get membrane fusion and releases contents to interior.

Physical Transfection Methods:

Micro-injection is very fine glass capillaries to inject DNA directly into cell. These are very expensive equipment.

Electroporation

- electric shock therapy for cells
- uses electric fields to open cells

- allows DNA to enter
- easy to do and reproducible
- requires more DNA and cells than other methods
- must control two things: maximum voltage and pulse duration

SV40 Transformation

Discovered as a contaminant in Rhesus monkey cells. They were once used to grow polio vaccine, monkey cells used to reduce chance of infectious undetectable virus, still fear of this today (anti-vaccine movement), some claim HIV was introduced in this way. In 1962 SV40 shown to cause cancer in rats and intensive studies launched to determine health risk. It became one of the most intensely active viruses. It is a common model now to study cell gene expression.

SV40 is used due to limited genetic information available. It relies heavily on cellular machinery for growth cycle.

Mechanism:

- No conclusive mechanism
- Likely caused by Large T gene product (LT)
- Thought to interfere with cell cycle inhibitor genes
- Rb, p53, ect., prevented from stopping cell cycle
- Transfected cell will proliferate 20-30 more times
- Then enter crisis stage
- A very few cells will become immortalized and overgrow
- Detection of virus
- Find specific antigenic viral proteins
- Viral DNA sequence detectable
- Viral mRNA present
- Virus can be rescued in some cases

Properties of Transformed Cells:

Growth properties of transformed cells are anchorage independent, loss of contact, inhibition. Transformed cells grow in suspension. They require a low serum quantity. Growth factor of the transformed cells is independent. These types of cells are immortal (loss of senescence). In the immortal cells the mutation are controlled by genes due to over expression of oncogenes. Shorter population doubling time, mutation in senescence control genes, due to over expression of oncogenes, and immortal does not mean malignant.

Genetic Properties:

- These cells have high spontaneous mutation rate
- Aneuploid, Polyploidy -
- Over expressed oncogenes , deleted genes

Structural Modifications are modified fttoste1tdh, cellular matrix, altered cell adhesion proteins and disruption of cell polarity, neoplastic, tumorigenic, and invasive.

Detecting Transformed cells are growth of multiple layers of cells on a monolayer. The cells grow under anchorage independent conditions.

- The normal cell lines have stable DNA content and transformed lines are aneuploid or heteroploid
- Good technique to ID transformed lines
- Use fluorescent dye that binds to DNA (P1)
- Gather data visually or via flow cytometry
- Analysis of RNA and protein content
- Look for characteristic gene product via Northern Blotting
- Get characteristic fingerprint of a cell type
- Antigenic Markers
- ID cells by screening for surface proteins
- Monoclonal antibodies are available for 'everything'
- Immunostaining or ELISA
- Cloning of Animal Cells

Cloned cell population is derived from single parental cell. Cloned cells are advantageous due to minimal genetic variability.

Cloning is a procedure used to isolate single cells in culture and then propagate single cells into a population of 'identical' cells. Various techniques are used in cloning of animal cells like:

- Dilution plating
- Cloning of adherent cells
- Use one of several possible substrata
- Cloning of suspension cells
- Soft agarose plating
- Uses of conditioned medium

Conditioned media is a special type of cloning media. It is enriched with AA and vitamins.

The chemical messengers play an important role in colony formation and called Colony Stimulating Activity Factors. They are provided by feeder layer and also from old media derived from confluent culture.

Conditioned media: Grow cells to 50% confluence, change media, grow cells for another 48 hrs, collect and filter sterilised media, and add to cloning medium 1:2. The cells which are able to grow in a particular medium have ability to adapt the factors in the conditioned medium.

Plating Efficiency:

Most of the primary cultures have low plating efficiency (near about 0.1%). Some established continuous cell lines are having better plating efficiency (over 10%). The Low Plating Efficiencies are found due to nutritional needs in low cell densities. The cells secrete messengers at high density which is required for survival and growth.

Plating efficiency can be improved by increasing Insulin and Dexamethosone quantity, using richest media, sometimes serum, FBS better than FCS or horse. Proper CO levels are the most important, and polylysine substrate or fibronectin helps some cell types.

Dilution Plating:

Prepare single cell suspension from monolayer culture.

For preparation of a single cell suspension take a dilute culture to 10 cells / ml and add 0.1 ml suspension to 96 well cloning tray.

Incubate for several weeks (colonies will form) and transfer colonies to flasks or dishes for further growth. The colonies will have 400-500 cells and trypsinize with 0. 1 m trypsin. They transfer cells into fresh media w/ 10% serum.

- Freshly sub-cultured cells are used in active growth to verify that cells are healthy and contamination free.
- Fresh explants are difficult to clone
- Almost always require feeder layer
- Cloning Suspension cells are good for hematopoietic stem cells or virally transformed fibroblasts
- Use semi-solid culture medium
- Often contains low concentration of agar or agarose
- Base layer of agar medium is placed into a Petri dish
- Thin layer of soft agar containing cells is overlaid
- Allows for microscopic observation
- Agar contains acidic and sulfated polysaccharides

Agar inhibitory to most cells due to inability of cells to anchor and inhibitory polyanions. It is not inhibitory to malignant or transformed cells and may use agarose as overlay (fewer polyanions).

Methocel is a viscous solution not as problematic as agar or agarose It is less dense than a gel. The cells would 'fall' to bottom and lie at the methocellagar interface. All are found on same focal plane.

Plant Cell Lines:

Plant cells respond to cryopreservation in a manner similar to other cells. The stage in the growth cycle from which they are harvested can affect their recovery, most optimum being late log phase. Also, cell density may play a role in recovery. Combinations of cryoprotective agents are more effective than agents used singly. The cooling rate is important, and in many cases, a two-step cooling process where the cells are held at —30 °C to -40°C for a period of time before cooling to liquid nitrogen temperatures, is beneficial. This process enhances the dehydration of the cytoplasm prior to freezing.

Viruses:

Most viruses can be frozen as cell-free preparations without difficulty and do not require controlled cooling. The exceptions are those viruses cultured in viable infected cells which require controlled cooling. Plant viruses can be preserved either in infected plant tissue or as purified virus preparations.

Equilibration:

The period of time between mixing the cryoprotectant with the cell suspension and the beginning of the cooling process is called the equilibration period. For most cells, equilibration should occur for at least 15 minutes, but no longer than 45-60 minutes. The cryoprotective agent may be toxic to the cells if the equilibration time is too long. Equilibration, which should take place at ambient temperature, allows time for the cryoprotective agent to penetrate the cells, with larger and less permeable cells requiring a longer equilibration period. During this period of time the cell suspension may be dispensed into vials and otherwise manipulated in preparation for freezing. The optimal equilibration time should be determined empirically for the cells being cryopreserved to maximize later recovery.

Rate of Cooling:

Once the cells and the cryoprotectant have been combined and dispensed into vials, the next step is to cool the suspension. The rate of cooling is important since it affects the rate of formation and size of ice crystals, as well as the solution effects that occur during freezing. Different types of cells may require different cooling rates, however a uniform cooling rate of 1°C per minute from ambient temperature is effective for a wide variety of cells. Generally, the larger the cell, the more critical slow cooling becomes. Most bacteria and spore-forming fungi will tolerate less-than-ideal cooling rates and can be frozen by placing the material at - 60°C for a period of time.

Despite the control applied to the cooling of cells, most of the water present will freeze at approximately —2°C to —5 T. The change in state from liquid to crystalline form results in the release of energy in the form of heat; this Is. known a$ the latent heat of fusion. Warming of the sample occurs until the equilibrium freezing point is reached, at which temperature ice continues to form. To minimize the detrimental effects of this phenomenon, under cooling must be minimized by artificially inducing the formation of ice. This can be accomplished by seeding the suspension with ice or some other nucleating agent, or by rapidly dropping the temperature of the external environment to encourage ice crystal formation.

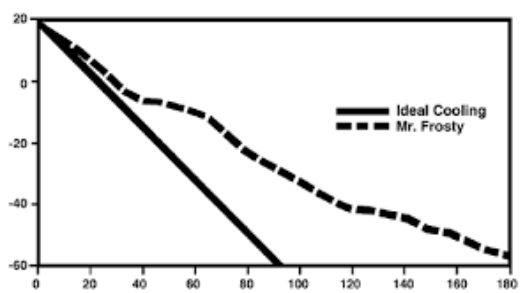

Fig1: The ideal cell cooling rate.

External Thread Internal Thread:

'Mr. Frosty' eliminates the need for direct immersion in an alcohol bath. This feature eliminates the potential for contamination due to wicking of the alcohol, as well as the presence of residual alcohol on the exterior of the vials

Storage:

When the sample has been frozen for 48 hrs, a vial should be thawed to determine whether the cells are viable and able to establish a cell population, i.e. survive the freezing procedure. The temperature at which frozen preparations are stored affects the length of time after which cells can be recovered. The lower the storage temperature, the longer the viable storage period.

Ultimate stability of frozen cells cannot be assured unless the material is maintained below -130°C , Some bacteria and spore-forming fungi may tolerate storage temperatures of -60°C to -80°C However, more fastidious cells, such as mammalian tissue cultures, must be maintained below -130°C

For ultimate security, living cells should he stored at liquid nitrogen temperatures. Improper handling of material maintained at cryogenic temperatures can have a detrimental effect on the viability of frozen cells. Each time a frozen vial is exposed to a warmer environment, even briefly; it experiences a change in temperature.

Storage systems should be designed to minimize exposure of stored material to warmer temperatures, as well as minimizing prolonged exposure of personnel during specimen retrieval.

Reconstitution (Thawing):

For most cells, warming from the frozen state should occur as rapidly as possible until complete thawing is achieved. To achieve rapid warming, place the frozen vial into a 37°C water bath. To minimize the risk of contamination during reconstitution, disinfect the external surface of the vial by wiping with alcohol-soaked gauze prior to opening. Immediately transfer the contents of the vial to fresh growth medium following thawing to minimize exposure to the cryoprotective agent.

Determination of Recovered Cells:

Methods used to estimate the number of viable cells recovered following freezing depend on the type of material preserved. Visual inspection alone can be deceptive, and although staining and dye exclusion are effective in determining the presence of viable cells for most mammalian cells, they do not indicate an ability to establish the cell population. For microbial cells, serial dilution and plate counts are effective in quantifying the population of cells recovered.

Step-by-Step:

1. Harvest cells from late log or early stationary growth. Scrape cells from the growth surface if they are anchorage dependent, Centrifuge broth or anchorage independent cultures to obtain a cell pellet, if desired
2. Prepare presterlised DMSO or glycerol in the concentration desired in fresh growth medium When mixing with a suspension of cells, prepare the cryoprotective agents in twice the desired final concentration.
3. Add the cryoprotectant solution to the cell pellet or mix the solution with the cell suspension. Begin timing the equilibration period.
4. Gently dispense the cell suspension into vials.
5. Begin cooling the cells after the appropriate equilibration time.
6. Remove the cells from the cooling unit and place them at the appropriate storage temperature.
7. To reconstitute, remove a vial from storage and place into a water bath at 37°C. When completely thawed, gently transfer the entire contents to fresh growth medium.

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