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Biopreservation of Cells for Use in Biotechnology and Cell Therapy

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Introduction

Today, technology has reached a point where organisms (bacteria, plant and animal cells) can be genetically engineered to produce specific macromolecules and perform complex chemical reactions. Hence, they are called "cellular factories."

Cellular factories have applications in:

- Biomedicine (*e.g.*, implanted insulin-secreting cells for the management of diabetes[1])
- Biotechnology (recombinant protein and enzyme production for pharmaceutical and food industries[2])
- Bioremediation (toxic waste and pollutant clean-up[3])
- Green chemistry (production of chemicals with minimum toxic bi-product generation)
- Alternative energy generation (electricity and hydrogen production by bacteria[4])
- Biosensors (*e.g.,* devices housing "canary cells", which can signal the presence of pollutants, viral agents, or toxic chemicals[5])
- Bioreactive devices (that can detect low concentrations of chemicals,[6] etc.)

At larger scales, plants are being used for the production (pharming) of antibodies, drugs, and vaccines.[7] It is interesting to see that many of the Grand Challenges for Engineering established by the National Academy of Sciences are in the areas where biotechnological applications based on cellular factories can make huge impacts: water purification, waste management, green energy production, carbon sequesteration, and engineering of better medicines. The success and widespread availability of all these methods and technologies depend on our ability to stabilize, and incorporate, these cellular factories into devices (such as bioreactive coatings, biosensors, or flow-through bioreactors), as well as to transport and store them until the time of use.[8]

In addition to being used to produce recombinant molecules, cells are also being used therapeutically for a wide range of diseases. The number of patients and diseases being treated therapeutically continues to grow, and cell therapies are being used to treat cardiovascular, neurological and hematological diseases.[9-12]

The National Institutes of Health has invested significantly in cell therapies as a treatment modality through the Production Assistance for Cell Therapy (PACT, <www.pactgroup.net>). This network of cell processing facilities supports the production of cells for therapeutic applications for investigators around the country. The University of Minnesota is currently one of three PACT sites; a listing of cell therapies currently in production at this facility is shown in Table 1. This listing

TABLE 1. Cell therapies in production or development at the University of Minnesota.

CELL TYPES	APPLICATIONS
Allogeneic UCB-derived regulatory T cells	Improving engraftment during bone marrow transplantation (BMT), allergy, autoimmune disorders, diabetes (Type I)
Allogeneic peripheral blood derived regulatory T cells	BMT, allergy, autoimmune disorders, diabetes (Type I)
Allogeneic UCB-derived natural killer cells	Treatment of cancer, BMT
Allogeneic peripheral blood derived natural killer cells	Treatment of cancer, BMT
Allogeneic mesenchymal stem cells	Improve engraftment during BMT
Autologous cardiosphere-derived cells	Heart failure
Bone marrow derived mononuclear cells	Acute myocardial infarction/congestive heart failure
Culture-expanded UCB	BMT
Skeletal myoblasts	Urinary incontinence
Dendritic cells	Brain tumors
Hematopoietic stem cells from UCB, bone marrow and peripheral blood stem cells	Multiple

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demonstrates the range of cells in use, and diseases currently being treated with cells.

The Importance of Biopreservation

To a large extent, the availability of technologies based on cellular factories depends on our ability to successfully stabilize and store them. Clearly, the ability to preserve the different bacteria, insect and plant strains created for research prevents genetic drift and reduces the cost associated with maintaining these specialized strains. Preservation is also critical for maintenance of native (wild-type) species. As concerns over biodiversity continue to grow, preservation of native species becomes even more critical. Another aspect of stabilization and preservation of cellular factories is somewhat futuristic: mining and colonization of other planets or asteroids.

The increasing demand for raw materials and the increasing earth population, combined with a slow but steady decrease in the habitable land mass (due to desertification and increasing sea levels), will eventually force mankind to look beyond the earth for alternatives. This may require transportation of specialized organisms (maybe extremophiles) to condition the atmosphere for human survival, for water purification, for the processing of natural resources, or for bioremediation in the new planets. The organisms developed for these purposes will need to be stable and preserved for very long periods of time, and survive hostile environments.

When used therapeutically, the ability to preserve cells is critical for clinical use. It is extremely common for cells used therapeutically to be collected at one site, processed at a second, and administered to a patient at a third site. Thus, the ability to preserve cells permits the transportation of cells between sites. The banking of umbilical cord blood (UCB) is the best example of this process. The ability to use short-term liquid storage followed by cryopreservation is integral to the UCB banking system worldwide. Cell therapies can require extensive safety and quality control testing before administration of the product to a patient. The ability to preserve cells permits completion of the testing before patient treatment. Cell therapy protocols may require culture or manipulation for days to weeks, and the ability to preserve cell therapies facilitates the coordination of the therapy with patient care regimes. Growth in the number of patients receiving cells to treat disease and the number of cell types used therapeutically only continues to increase.

In the near future, the number of patients that can be treated will be limited by the capacity of cell processing facilities. The ability to cryopreserve cells will permit cell therapy products to be continuously produced and then stored until the patient needs them. This process permits the development of a "manufacturing paradigm" for cell therapies, thereby maximizing the number of products that can be produced at a given facility.

The objective of this article is to summarize fundamental concepts in cryopreservation. These fundamentals can be used to help in the development of cryopreservation protocols as well as improving outcome for existing protocols.

Elements of a Preservation Protocol

The components of a cryopreservation protocol are given schematically in Figure 1. These elements include: prefreeze processing, introduction of a cryopreservation solution, cooling protocol, storage, warming, and postthaw assessment. We will present a brief overview of these components and the manner by which improper design of the component can result in a poor outcome of the preservation protocol. Further information on the fundamentals of preservation and protocol development can be found at our website.[13]

Prefreeze Processing

Not surprisingly, the manner by which a cell is handled prior to freezing may influence its ability to survive the stresses of freezing and thawing. Cells can be subjected to a multitude of processes prior to cryopreservation including, but not limited to: culture, genetic modification, and selection of subpopulations. Any of these processes can non-lethally stress the cells (shear stresses, nutrient/oxygen deprivation, shifts in membrane composition), but may compromise the ability of cells to survive the stresses of freezing and thawing. For example, umbilical cord blood is collected in a hospital and shipped in the liquid state to a cell processing facility, where it is red blood cell depleted and then cryopreserved.

Studies have demonstrated that the liquid storage conditions (duration of storage, temperature, cell concentration, storage solution) influences the ability of the cells to survive the stresses of freezing and thawing.[14-16] Pre-freeze processing should be evaluated for its influence on post-thaw

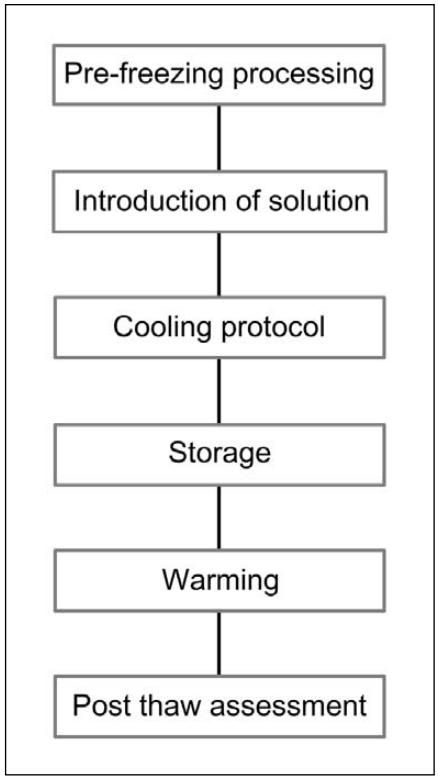

FIGURE 1. Elements of a cryopreservation protocol.

recovery. Monitoring cells for early signs of apoptosis or shift in metabolism to stress pathways may also be helpful in determining if a pre-freezing processing protocol may be potentially harmful to the cells.

Formulation and Introduction of a Cryopreservation Solution

Modern cryopreservation started when Polge and colleagues observed in 1949 that the addition of glycerol to a solution permitted the survival of sperm.[17] Since then, certain additives (cryoprotective agents) have been used to improve the ability of cells to survive the stresses of freezing and thawing. The most commonly used cryoprotective agents are glycerol and dimethylsulfoxide (DMSO). These additives have been shown to act through a variety of mechanisms. The addition of organic molecules reduces the concentration of salt at a given subzero temperature,[18] influences the growth and structure of the ice phase,[19] or stabilizes the cell membrane.[20-22]

The principal component of a cryopreservation solution is culture media or a balanced salt solution. This solution is supplemented with cryoprotective agents (DMSO and glycerol). Some cell types also benefit from the addition of proteins to the solution.

It is noteworthy that cryopreservation solutions are not physiological. For example, a 10% DMSO solution is approximately 1.4 Osm. When transferred from an isotonic solution (270–300 mOsm) to a 10% DMSO solution, cells exhibit a rapid efflux of water and, slowly, the DMSO from the surrounding solution permeates the cell membrane. Both the rate of volume change and the absolute volume change experienced by the cell can produce cell lysis.[24] These same osmotic stresses can be observed when the cryopreservation solution is removed. Specifically, cells will experience a rapid influx of water followed by a slow efflux of DMSO. Cells are much more sensitive to lysis upon expansion, so post-thaw DMSO removal protocols are critical for preventing cell losses.

Cell losses can result not only from the introduction or removal of CPA solutions, but also from exposure to the solution over time. This mechanism of cell loss is most commonly known as biochemical toxicity. The sensitivity of one cell type, hematopoietic stem cells (HSCs), to DMSO has been studied.[25] During the pre-freeze and post-thaw periods, exposure time of the cells to DMSO is minimized. Specifically, all reagents (cells and cryopreservation solution) are chilled and the freezing process must start within 15 minutes. As demonstrated with HSCs, cooling the cells and minimizing time of exposure can reduce cell losses resulting from biochemical toxicity.

Current strategies for introduction and removal of cryopreservation solution from cell suspensions are time consuming, labor-intensive, and result in significant cell losses. For the introduction of a cryopreservation solution, cells are centrifuged to form a cell pellet at the bottom of a container (bag or test tube). The supernatant is removed and replaced with a cryopreservation solution. For the removal of a cryopreservation solution, the same process is repeated (centrifugation followed by removal of the supernatant) but a wash solution is added to the cells and the process is typically repeated to minimize the presence of residual solution. The entire removal process takes 1.5 to 2 hours in the clinical lab.

Cell losses can occur due to mechanical stresses on the cells during both centrifugation and expression of the supernatant. Further, the centrifugation process requires significant intervention of an experienced and skilled operator in order to minimize losses. Antonenas and colleagues quantified losses of 27–30% of nucleated cells resulting from post-thaw washing of UCB.[26] Recently, a microfluidic device has been developed that reduces the time, cell losses and semi-automates the process of removing a cryopreservation solution.[27]

Cooling Rate

The strong influence of cooling rate on post-thaw survival has been documented for a variety of cell types.[19] The cooling of the cells can be performed using a controlled rate freezer or through mechanical freezing. During controlled rate freezing, reducing the temperature of the freezing chamber influences the temperature of the sample to be frozen. During mechanical freezing, the cooling of the sample is not controlled, but the sample is passively cooled and the cooling rate decreases with time as the sample cools to the temperature of the mechanical freezer.

For either method of cooling, the temperature at which ice forms in the extracellular solution is critical. Nucleation of ice in the extracellular solution results in removal of water from the solution in the form of ice and a corresponding increase in extracellular concentration.[18] Studies by Toner and colleagues demonstrated that the temperature at which ice forms has a profound influence on post-thaw viability.[28] Specifically, decreasing the temperature at which ice is formed in the extracellular solution increased the fraction of cells that are damaged for a given cooling rate. Controlled rate freezing protocols may control the temperature at which ice forms in the extracellular solution by inserting a rapid cooling step, followed by rapid warming as a "seeding step."[29] This step does not insure that every sample in the controlled rate freezer forms ice in the extracellular solution at the same temperature, but it increases the likelihood that it will.

Freezing samples with a mechanical freezer does not provide the opportunity to influence the temperature at which ice forms, but some cell types tolerate this approach. Limitations in our ability to monitor temperature during the freezing process for each sample being frozen hampers our ability to develop optimal protocols for cell freezing, and to develop effective methods of eliminating samples whose actual freezing protocols have deviated from optimal protocols.

Storage

Storage conditions will influence product stability/shelf life of a cryopreserved product. Two major factors influence the stability of a frozen product during storage: the composition of the cryopreservation solution and the biological activity of the cells. Cryopreservation solutions are complex, multi-component mixtures that do not freeze at a single temperature, but over a range of temperatures.[18] The highly concentrated, unfrozen solution that forms after the seeding of the extracellular solution does not freeze completely until the system reaches the eutectic temperature. For a 10% DMSO solution, the eutectic temperature is approximately -70°C.[30]

Other cryopreservation solutions that are commonly used have eutectic temperatures as low as -120°C.[31] Storage of a product at or near the eutectic temperature implies that the extracellular solution is not fully solidified, and the cells will be surrounded by high concentration solutions which can in turn influence post-thaw recovery.[32]

Stability of a frozen and stored cell therapy product is also influenced by cellular activity. Much of the cells' activity, such as water transport, is minimal for temperatures below -40°C. However, enzymatic activity of cells persists to very low temperatures, and this activity can influence post-thaw recovery. Tappel studied the activity of common intracellular enzymes at low temperature[33] and observed that there is a threshold temperature below which the enzymatic activity is suppressed. The actual threshold temperature depends upon the enzymes present, but storage below -150°C is typically recommended.

More recently, Fowke and colleagues[34] observed that post-thaw apoptosis levels increased when mononuclear cells from peripheral blood were stored at higher temperatures (-70°C). Thus, storage of cells at temperatures above that of liquid nitrogen may reduce the shelf life of the product.

Warming

Warming can be just as critical to cell survival as cooling. The same dangerous chemical and mechanical environment that is observed during freezing is present during warming. As with cooling, the cells are exposed to very high extracellular concentrations during warming, and those concentrations can be damaging.[19] In addition, the cells can be subjected to recrystallization damage as very small ice crystals present in the cells during cooling may have time to grow during warming. The optimum warming protocol is influenced by the cooling protocol used.[35] For conventional controlled cooling rate freezing over the range of cooling rates used for most cell types (1-30°C/min), optimal warming protocols should be as rapid as possible (>200°C/min).

High warming rates are most commonly achieved by agitating the sample in a warm water bath until a significant fraction of the visible ice crystals have melted. Higher warming rates can also be achieved by increasing the temperature of the warm water bath used for thawing. However, using higher bath temperatures must be evaluated carefully in order to prevent damage to the cells resulting from exposure to supraphysiological temperatures.

Post-Thaw Assessment

Accurate and meaningful measures of post-thaw assessment are critical to the development of effective preservation protocols. It is a very difficult process and a common source of problems when developing cryopreservation protocols. Viability assays can be divided into different categories: a) physical/membrane integrity; b) metabolic activity; c) mechanical activity (attachment, contraction); d) mitotic activity (proliferation assay); and e) transplantation potential.[36]

Each of these assays provides important information and, typically, use of one assay is not sufficient. For example, numerous studies have measured high levels of membrane integrity for frozen and thawed hepatocytes,[37-39] but unless these cells attach to a surface and exhibit metabolic functions, the cells are not useful. Therefore, post-thaw measures of hepatocyte function will frequently involve assays for a variety of functions including the synthetic and detoxification functions of the cells.

Post-thaw assessment presents specific challenges that differ from determining the viability of a cell that has not been subjected to freezing and thawing. First of all, cells that have been frozen and thawed, and are still intact, have undergone extensive dehydration that may leave the cell membranes transiently leaky.[36] These cells have also experienced suppression of metabolic activity, and there can be a delay between thawing and the resumption of normal metabolic activity.[40] Finally, post-thaw apoptosis has been observed in several cell types.[41,42-44] Therefore, the viability of cells that have been frozen and thawed may vary with time in post-thaw. Care must be used in timing the post-thaw assessment.

Summary

The ability to preserve cells is critical to a wide range of industries. Cryopreservation protocols can be developed based on scientific principals and include the formulation and introduction of cryopreservation solutions, controlled rate freezing, storage, warming and post-thaw assessment. Each element of the protocol is important and can have a strong influence on post-thaw recovery. Further improvements in our ability to preserve cells will require development of both the fundamental science of preservation and the enabling technologies.

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