DEVELOPMENT OF A DIMETHYL SULFOXIDE – FREE CELLULAR PRESERVATION TECHNIQUE FOR HUMAN MESENCHYMAL STEM CELLS

by

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Abstract

As the use of living human cells as the therapeutic component of modern drugs becomes more prevalent, the need to store and transport those cells is increasing. Currently the most common methods for maintaining these living cells for extend periods of time, months or years, is through cryopreservation at ultra low temperatures requiring the use of possibly toxic and cellular damaging chemicals. This study examines alternatives to the use of dimethyl sulfoxide (DMSO), and subzero temperature storage. Two different DMSO-free solutions, using materials already prevalent in the medical industry, were tested and shown to be viable alternative cryopreservation solutions. A method of freeze-drying (lyophilizing) and rehydrating living cells was tested, and shown to be reproducible, but resulted in alterations to the cells that prevented continued culture. Though not conclusive, the data here demonstrate that solutions and methods of long-term storage of living cellular material without the use of DMSO is possible, and storage at or near room temperature may be promising.

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INTRODUCTION

Literature Review

The idea of treating disease with living cells continues to increase in popularity with the biotechnology community and the medical field at large. Various cellular therapies are being explored in countries all over the world, and in every case there will be a critical component to these new therapies, that the cells cultured outside of the patient's body must be transported to the patient for administration. Particularly in the case of allogeneic therapies, like human mesenchymal stem cells (hMSCs), this often requires an extended period of storage and transport. Additionally, for many of these new treatments to become cost effective and economically viable they will need to be made in large quantities, stored, and shipped at very low temperature. The development of reagents and methods to maintain these cells at or near room temperature would dramatically reduce the cost and complexity of the current cell therapy industry.

Historically the primary need for storing living cells has been for research or other bio-production where the living cells were not being introduced to humans. In most cases cryopreservation utilizing the industrial solvent dimethyl sulfoxide (DMSO), or by vitrification of a small numbers of cells, has been sufficient. The number of cell therapies in the clinic has increased dramatically from ~683 in 2003 to over 27,000 in 2018, with just over 800 clinical trials specifically involving hMSCs (ClinicalTrials.gov 2018). With this increased need for clinically viable, long-term storage of cells there is a growing need to improve the methods used to store, transport, and administer these cells. Though there are many anecdotal accounts of long-term topical use of dimethyl sulfoxide (DMSO), as of 2018 the only FDA approved use is

for treating interstitial cystitis. There are currently no known long-term side effects of DMSO exposure, however patients have reported a variety of symptoms including dry skin, erythema and pruritis, urine discoloration, halitosis, agitation, hypotension, sedation dizziness, and a garlic like taste in the mouth and odor of the skin (Brayton 1985; Prior *et al.* 2000). The discovery of alternative DMSO-free methods for extended preservation of cell-based therapeutics could reduce the stressful side effects on patients. Additionally, there is evidence that DMSO can have negative impacts on several types of human cells. A recent study demonstrated that solutions of 5-10% DMSO (v/v) increased cell death in peripheral blood mononuclear cells (PBMCs) as well reduced cytokine production and proliferation in various lymphocytes (de Abreu Costa *et al.* 2017). There are also other incentives to explore alternative to DMSO as the sole cryo-protective agent. While the amphipathic polar nature of DMSO is ideal for inhibiting crystal formation in water, it will never provide the kind of protection that cellular protein will need in the absence of an aqueous solution. Lyophilization, freeze drying, is a common technique for storing a variety of proteins, and can provide greatly extended shelf life as opposed to refrigeration or freezing while negating the need for specialized equipment for storage and shipment. This study aims to generate data that can be used to develop a cryopreservation solution that is effective at preserving viable therapeutic cells in the absence of DMSO, does not cause adverse alterations to the cells, is safe for use in humans, and has potential to stabilize the cellular proteins in a dehydrated environment.

Cryopreservation is essentially the use of ultra-low temperatures to halt or substantially reduce the biochemical damage done over time by enzymatic or chemical activity in a biological system. As our understanding of the biological world expands the need for safe and reliable cryopreservation of cellular material is also expanding. Cryopreserved cells are now being used

not just to preserve research samples, but in developing therapies, biobanking, and clinical manufacturing. Often there is a requirement for the cells to be stored for a period of time prior to being received by the patient. In some cases the cells may need to be shipped great distances or stored for long periods of time. In these instances it is usually advantageous to cryopreserve the cells to provide a more consistent final product. There can be a number of strategies for achieving a viably cryopreserved final product, but currently each method has significant drawbacks. These cryopreservation strategies can be described as slow freezing methods, or rapid freezing methods.

The goal of a rapid freezing strategy is to promote water vitrification as opposed to crystal formation. In this process water in the intracellular as well as the extracellular environment freezes at a sufficiently rapid rate that crystals do not have time to form. Cells are then protected from the physical damage of ice crystal formation, and there are no dramatic fluctuations in pH or osmolality due to water-soluble materials precipitating out of solution. Vitrification has been shown to provide superior results with particularly freeze sensitive cell types, such as embryonic stem cells (Hunt and Timmons 2007). DMSO does promote vitrification, in that it reduces the freezing point thus promoting vitrification opposed to crystal formation. However, DMSO alone is rarely enough to protect particularly sensitive cellular material and often other more toxic compounds like ethylene glycol are added (ESI 2005). This method of cryopreservation can be complex, require specialized equipment, and is rarely desirable in a therapeutic process as chemicals like ethylene glycol have toxic side effects and vitrifying larger samples is challenging.

Slow freezing is the most common alternative to rapid freezing and vitrification. In this method, a variety of molecules or compounds are utilized, either alone or in various

combinations, to prevent cellular damage throughout the freeze and thaw process. These molecules or compounds can be collectively referred to as cryoprotective agents (CPAs), and can be further described as acting on the intracellular, extracellular environment, or acting both internally and externally to the cell. CPAs can provide protection against freezing damage in a variety of ways. Sugars, like trehalose, can have a stabilizing effect on cell membranes and proteins by associating with the surface of these structures, which provides stability and shear protection as ice forms around them (Hubel 2012). Small soluble molecules like DMSO provide a colligative effect (Hubel 2012). This is a phenomenon where the properties of the complete solution have different properties than that of the individual components, particularly with regard to boiling temperature and freezing temperature. These CPAs provide protection by reducing the temperature at which ice begins to form, which promotes vitrification and reduces the time metabolic processes are active as ice is formed. This type of cryopreservation is much more broadly applicable to various mammalian cell types and larger volume formulations than vitrification. There are however still challenges with this method. DMSO is almost ubiquitously the CPA, and often attributed to excessive cellular damage and signs of uncontrolled differentiation (Katkov *et al.* 2006). Effective cryopreservation solutions often must be custom tailored to the cell type, or are extremely complex in their formulation.

Early strategies for cryopreservation typically utilized the small sugar alcohol molecule glucose, but few cell types survive well utilizing this approach alone (Norris *et al.* 2006). Other methods include the use of slightly larger sugar molecules, such as the dissacharide sucrose, to stabilize the external feature of a cell during low temperature freezing (ESI 2005). This could be described as a structural or stabilizing method of cryopreservation. As the cell is frozen the sugar molecules bound to the surface of the cell provide structural support and help the cell resist

the mechanical forces imposed upon it during ice formation. While this method can work well for certain cell types, the viability of the cellular material post thaw is typically lower than other methods. More recent methods are typically focused on a colligative method of cryopreservation. A small soluble molecule capable of transversing cellular membranes (absent the active transport necessary for sugars) is used to increase the number of dissolved particles both within the cell and the external environment. As an increased number of particles are dissolved in water the temperature at which that water will freeze is depressed. This can be described in the following equation, $\Delta T = -1.86\ x\ b$ where ΔT is the freezing point depression and b is the molality of the solution. The theory of this technique is that if the temperature at which ice begins to form is reduced to a point that the majority of cellular processes have already ceased to function, then the extent of damage will be reduced once ice begins to form. The most common molecule of choice is DMSO, which is typically used at a concentration of approximately 5-10% of the total volume.

Problems have been documented with the use of DMSO use in conjunction with living cells. Embryonic stem cells have been shown to exhibit reduced levels of the transcription factor Oct-4, indicating a reduced level of pluripotency (Katkov *et al.* 2006). The failure of at least one phase III clinical trial implicated DMSO exposure with reduced viability and functionality (Galipeau 2013). There have also been patient side effects associated with DMSO exposure include nausea, headache, and skin rash as listed by the U.S. Food and Drug Administration (FDA 2011). Additionally, therapies including exposure to DMSO are widely reported to be associated with a garlic-like taste in the mouth, and patients are reported to emit a strong garlic-like body odor. Though these adverse events (AEs) may not represent life threatening risks, the long-term effects of DMSO exposure are not currently well understood. DMSO also has the

added risk of being a "carrier" molecule, in that it has the ability to permeablize cell membranes, thus allowing molecules to enter the cell that would ordinarily not be able to pass. Currently the only FDA approved human use of DMSO is for the treatment of interstitial cystitis, a bladder condition (FDA 2011). DMSO has also been shown to alter cell membranes at concentrations as low as 2.5% or even completely destroy cell membranes at concentrations exceeding 25% (Gurtovenko and Anwar 2007).

Fundamentals of Cryobiology

The first step in developing alternate cryopreservation solutions is to understand the mechanisms by which cells are damaged during freezing and subsequent thawing. As a cell freezes, and the water freezes surrounding it, several factors combine and have a negative effect on the cell and the microenvironment. The most obvious is ice crystal formation. As the solution cools below the freezing point the bonds between individual water molecules become stronger and they orient themselves into a crystalline structure. As this happens water expands, leaving less room for cellular components, altering cellular shape, and possibly damaging the internal cytoskeletal system of the cells, or even causing lysis. A second, and perhaps greater risk to the cell, is the transition of previously soluble material to an insoluble phase. As water freezes, the ability to dissolve polar molecules is lost and these molecules become increasingly more concentrated in any remaining liquid water or polar solvent. For the same reason, salts and buffers concentrate in reduced liquid volumes. This can dramatically affect the pH and alter chemical interactions. Additionally, many of these same factors can come into play during the thawing of cryopreserved cells. Small molecules used to preserve the cell during freeze can lead to greater osmotic pressure inside the cell during thaw. All of these factors, and perhaps others

that are not yet understood, often result in decreased viability, reduced vitality, apoptosis, or uncontrolled differentiation once cryopreserved cells are thawed and returned to culture (Hubel 2012)

There are a number of approaches to mitigating the adverse effects of cryopreservation. These strategies can be described as being intracellular, extracellular, or a combination of both. The use of DMSO could be described as being a combination intracellular and extracellular approach. In this case the small polar nature of this molecule allows it to readily intercalate with and pass through the cell membrane. As with similar small soluble molecules, like glycol and methanol, this increase in the number of solutes will decrease the eutectic point, or temperature at which a liquid will transition from liquid to solid. To give an example of this, a 50% mixture of water and DMSO will remain liquid nearly to -80°C (McKim and Strub 2008). A second strategy, discussed earlier, is to utilize larger molecules, such as trehalose or dextran, which will primarily bind to the cell surface thus stabilizing the plasma membrane during the freezing process. A third method mentioned is vitrification, where water is essentially frozen at such a rapid rate ("snap freeze") that the crystalline ice structure does not form. Cells are then protected from the adverse effects of expanding ice and the length of time they are exposed to destructive chemical and biological process is greatly reduced. This technique can be complex and requires potentially toxic components such as ethylene glycol, but has shown promising results in the freezing of fragmented cell colonies (Hunt and Timmons 2007). Other groups have demonstrated a degree of success utilizing propanediol, DMSO, and polyvinyl alcohol (Wang *et al.* 2009). In either case the technique of vitrification required very small sample volumes, ≤ 1 mL, to be minimally protected and exposed to liquid nitrogen. It may be possible to expand this technique using colder cryogenic liquids, or heating a sample with a laser while super-cooling

the external environment and subsequently turning off the laser, but all of these techniques are complex, and likely to only be effective on small volumes. Thus the development of a large-scale process to vitrify therapeutic living cellular material is unlikely.

Other Molecules with Cryoprotective Potential

Several other molecules are gaining interest for use as potential CPAs. The possible use of neuropeptides such as glucose-dependent insulinotropic peptide (GIP), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are three examples of potential new CPAs. All three have been successfully incorporated into cryopreservation protocols containing DMSO and/or trehalose in previous studies (Briquet *et al.* 2013). These neuropeptides are of particular interest due to their ability to suppress apoptotic pathways. The anti-apoptotic role of PACAP has been observed in various cellular models where the expression of the mitogen-activated protein kinase pathway has been suppressed (Gasz *et al.* 2006) or expression of bcl-2 expression has been increased (Kim *et al.* 2008). Further research into the use of these or similar neuropeptides may reduce or eliminate the need for animal origin serum or human serum albumin while providing an additional anti-apoptotic effect.

Other molecules of interest include Pluronic F68 block copolymer, also known as poloxamer 188 (F68), Proline, and Ectoin. All of these molecules have been utilized in previous cryopreservation studies with varying degrees of success. F68 has been utilized in conjunction with DMSO and FBS to show an increase in the number of viable hMSCs as well as their proliferation post cryopreservation (Dogan *et al.* 2013). Proline and Ectoin have been used together in conjunction with methylcellulose in a DMSO-free cryopreservation solution to freeze hMSCs with promising results (Freimark *et al.* 2011). Proline may be of particular interest

because it is a naturally occurring biomolecule and has been shown to bind to and stabilize proteins at cryogenic temperatures (Pemberton *et al.* 2012).

Many organisms in nature have developed natural mechanisms to cope with subzero conditions, in some cases maintaining normal cell function even after thawing from a completely frozen state. Certain frogs and salamanders have been observed to utilize glycerol to survive subzero temperatures, where other animals are able to accumulate sorbitol, ribitol, erythritol, threitol, and/or ethylene glycol within their tissues to survive freezing conditions (Brockbank *et al.* 2011). The ability of certain species of tree frogs to avoid freezing damage is attributed to the maintenance of increased levels of glucose within their tissues (Brockbank *et al.* 2011). Glucose has long since been a popular CPA for mammalian cells, largely due to the fact that it is readily taken up by receptors on the cell surface, however, it is quickly metabolized by most mammalian cells and thus does not reach sufficient concentrations to be an effective CPA. One possible alternative is the non-metabolizable derivative, 3-*O*-methyl-D-glucose. An earlier study showed that this form of glucose was still absorbed by keratinocytes and offered significant protection during cryopreservation (Norris *et al.* 2006). Trehalose is another example of a molecule found in a variety of organisms and also shows considerable promise as a CPA. Although there are challenges with inducing cells to take up trehalose, this disaccharide has become one of the most studied molecules for its potential as a cryopreservation agent. Studies have been conducted, with some success, utilizing the novel amphipathic membrane permeabilising agent PP-50 to increase trehalose uptake in the human SAOS-2 cell line (Sharp *et al.* 2013). Additionally, and with slightly greater success, trehalose uptake has been improved with long-term exposure during cell culture (Campbell and Brockbank 2012).

Cell Therapy

In recent years the interest in developing novel treatments for a wide variety of ailments utilizing living, metabolically active cells has become increasingly popular. Often referred to as cell or cellular therapies, the idea of introducing living cells into a patient for regenerative purposes is being explored to treat a wide variety of conditions such as Parkinson's Syndrome, Ischemic Stroke, and post-operative care for joint replacements (Dogan*et al.* 2013).

Whatever the use, these therapeutic cells can be broken down into two major distinctions or classes of therapy. Either they are autologous, cells derived directly from the same patient, or they are allogeneic, cells derived from another source. In an autologous therapy the patient would donate his or her own cells. These cells would then be expanded or altered outside of their body. Typically this is to either increase their number to treat a deficiency in the patient, or to alter their function to provide a new therapeutic potential. The cells are then reintroduced into the patient from whom they were originally extracted. Autologous therapy has the advantage of little risk of rejection because the cells originate from the same patient, and thus are typically not recognized as "non-self." Most autologous therapies are administered with fresh cells shortly after their harvest. Thus prolonged storage is not necessary, and if shipping is required 2-8°C refrigeration is usually sufficient. Also, these types of treatments often only require single administration so prolonged storage of a product in bulk is often not relevant.

In allogeneic therapy, the therapeutic cells are derived from someone other than the patient. In this case, cells or tissues are extracted from one donor. The cells of therapeutic interest are then isolated, expanded, and often stored in some way prior to reintroduction into a different patient or patients. This therapy has the disadvantage that, due to the therapeutic cells being derived from a different individual than the patient, there is considerable potential for

immunological rejection. The great advantage of this type of therapy is that cells can be produced in massive quantities over considerable lengths of time and stored for future use, as patients require them.

Human Mesenchymal Stem Cells

Currently one of the most promising candidates for use in cell-based therapy is the human mesenchymal stem cell (hMSC). According to clinicaltrials.gov, there are more than 800 clinical trials worldwide currently being conducted involving these cells, 150 in the US alone (ClinicalTrials.gov 2018). At least some of this popularity can be attributed to these cell's ability to suppress damage caused by inflammation in many organ systems with a minimal need for complex targeting strategies. When injected locally or intravenously, hMSCs appear to broadly suppress inflammation and promote healing many different tissues (Bernardo and Fibbe 2013). Coupled with the fact that many allogeneic therapies using these cells have shown promising results, they are a becoming a very attractive potential therapeutic source.

Lyophilization (freeze-drying)

Lyophilization is another interesting possibility for long-term storage of cell-based products. Lyophilization is often referred to as freeze-drying, and is essentially a method by which all, or nearly all, of the water in a solution is removed through a combination of freezing and reducing the surrounding atmospheric pressure. In this manner, solid water is induced to sublimate directly into the gas phase. The remaining dehydrated material is mostly chemically and biologically inert. If living cells can be sufficiently protected throughout this process it may

be possible to preserve them at much higher temperatures compared to cryopreservation alone, and thereby greatly reducing the complications of transportation and storage.

Some success in the lyophilization of hMSCs has already been reported. Zhang et al. (2010) have reported recovery of cells with 69.33±13.08% viability post lyophilization and storage at room temperature for two hours (Zhang *et al.* 2010). These results seem very promising, but did not demonstrate any functional properties of the cells post lyophilization. In the same study trehalose loading was examined. It was determined after incubating hMSC culture for 24 hours in growth medium containing 10, 20, 50, 100, and 200 mmol/L solutions of trehalose that at 100 mmol/L solution provided the optimal results in terms of calculated uptake of trehalose inside the cell (Zhang *et al.* 2010).

Other methods have also been attempted to stimulate trehalose uptake in human cells. In a study on hematopoietic stem and progenitor cells (HPC) adenosine triphosphate (ATP) was used to stimulate an endogenous cell surface receptor, termed P2Z, to increase the efficiency of trehalose loading (Buchanan *et al.* 2010). In this study a solution of 5 mM ATP and 200 mM trehalose was used to induce pore formation in the cell membrane and increase the uptake of trehalose. Cell were then lyophilized in a FTS DuraStop MP/DuraDry MP freeze-dryer and stored at room temperature four weeks. The cells were subsequently rehydrated using sterile water and plated in methylcellulose. When subjected to differentiation the lyophilized cells performed comparably to freshly isolated cells. This suggests that at least some cell types may tolerate trehalose loading and lyophilization and retain many functional attributes.

With the increased demand for cell-based treatments there is a growing need for long-term storage of living cellular allogeneic products. Cryopreservation is still the leading method for this type of storage, but due to the potential for toxic or adverse side effects, to the patient or

the therapeutic cells, the conventional use of DMSO is not an ideal solution for clinical applications. Additionally, DMSO alone is only effective when dissolved in an aqueous solution and frozen to cryogenic temperatures. This makes DMSO a very unlikely candidate for use at or near room temperature storage. If a cell therapy product could be formulated stably for an extended period of time it would dramatically reduce the cost and complexity of storing and shipping that product. Many potential CPAs have been and continue to be studied, but a viable and broadly applicable alternate formulation that would allow simple low-cost storage and distribution has not yet been discovered.

Study Rationale

The aim of this research is to further the development and understanding of alternatives to DMSO based cryopreservation and long term ultralow temperature storage. Having alternative cryopreservation solutions to DMSO would provide more options to researchers and pharmaceutical developers. The removal of a potentially toxic component may make future drug approval simpler, and reduce the potential side effects on patients. If the same solution could be used to maintain viable therapeutic cells at normal refrigeration or room temperatures the cost of storage and transport of those cells would be greatly reduced. A cell therapy drug that could be maintained at room temperature or at 2-8°C would be less costly to maintain a larger inventory and help facilitate the practicality of larger scale manufacturing. Untimely, a DMSO-free cell therapy that does not require ultralow temperature storage would be more accessible and affordable for a broader range of patients.

Objectives

Objective 1: Evaluation of a DMSO-free cryopreservation medium

The first objective of this study was the evaluation of two DMSO-free cryopreservation media or CPMs. These CPMs used components already commonly used in the medical industry, such PLASMA-LYTE A, hetastarch, human serum albumin (HSA), polyvinylpyrrolidone (PVP) and trehalose. Samples of hMSCs cryopreserved in these solutions were created after a period of cell culture and trehalose loading either by ATP mediated loading, spontaneous pore formation, or extended culture. These samples were then analyzed for viability and ultimately one method and solution was selected for further evaluation. That method was then repeated with additional controls, and additional samples were generated. Those samples could then be further examined

for viability, the presence or absence of surface markers, and subsequent secondary cell culture in parallel with several controls.

Objective 2: Evaluation of lyophilization with DMSO-free media as an alternative long-term storage method for hMSCs.

The second objective of this study was the evaluation of lyophilization as a method of stabilizing viable hMSCs at refrigeration temperatures opposed to ultra low temperature storage. The same samples generated using the two DMSO-free cryopreservation solutions and trehalose loading techniques were subjected to lyophilization. Those samples were then analyzed for viability. Using that data along with the data generated from cryopreserved cells with the same formulations, a single method was selected for further analysis. The process was then repeated with additional controls and subjected to surface maker analysis and evaluated for growth potential.

MATERIALS AND METHODS

Preliminary Cell Culture Evaluation

The primary goal of this phase was to establish the culture condition and methods that would be used in the experimental phase of this project. The Cell Therapy Process Development group at Lonza Walkersville Inc. provided the hMSCs used throughout this project. Many of the materials and reagents originally used to isolate and culture these cells were developed as part of a proprietary process for specific clinical requirements. Thus, replicating that process precisely was not practical, and the performance of these cells using readily available materials and reagents needed to be established. The hMSCs used were previously isolated from human bone marrow collected as part of a FDA approved clinical trial and later donated for research use. Cells were cultured for two passages, and cryopreserved at ~10 million cell/mL in a 10% DMSO solution to produce a master cell bank (MCB).

To begin the preliminary cell culture evaluation a master cell bank was thawed using a Genesis Plasmatherm at 37.0°C for 4 minutes, then resuspended into cell culture media consisting of Alpha MEM from Lonza (12-169F) supplemented with 15% v/v% fetal bovine serum (FBS) from HyClone (SH30071.03). Cryopreservation media was removed by centrifugation at 500 x g. These cells were originally cultured in an alternative proprietary media. To determine the appropriate conditions and culture schedule two short expansions were performed. The first evaluated use of Alpha MEM supplemented with various concentrations of FBS v/v%, 5%, 10%, 15%, and 20%. In all cases the cells were seeded at 1000 cells/cm^2 into one Corning T-225 flask (3293), placed in an incubator at 37.0°C with 5.0% CO_2, and evaluated microscopically daily for 7 days, with one media exchange on the 4^{th} day. After 7 days, cells in the 15% and 20% FBS groups visibly had formed larger clusters and were more confluent than

those in the 5% or 10% FBS flasks. This culture was terminated and a new T-225 flask was seed at 8000 cells/cm^2 with Alpha MEM supplemented with 15% FBS v/v%. This flask was observed daily and media was exchanged on day 4. On day 7 the culture was judged to be > 95% confluent. The flask was harvested using 0.05% Trypsin-EDTA (Gibco part 25300-054) and the total viable cell (TVC) yield was determined using a Chemometec automated cell counter NucleoCounter, Model NC-200 using the aggregated cells assay and default gating. The culture timing and TVC yield were determined to be adequate for the purposes of these experiments. From this point forward all cell culture was conducted under these conditions. Cells were seeded into T-225 flasks at 8000 cells/cm^2, using Alpha MEM supplemented with 15% FBS, a media exchange was performed on day 4, and cultures were harvested on day 7 using 0.05% Trypsin-EDTA. Any centrifugation required for removal of supernatant or concentration of cells was performed at 500 x g.

Experiment #1: Evaluation of DMSO-free cryopreservation media trehalose loading, lyophilization, and rehydration

The initial goal was to determine one method of trehalose loading and one DMSO-free CPM that could be used in further experiments. Experiment #1 was designed to evaluate three potential strategies for loading trehalose as well as evaluate two different DMSO-free cryopreservation solutions that could potentially be used for lyophilization. Three different methods of trehalose loading were tested, ATP mediated loading, Arm A; loading by spontaneous pore formation, Arm B; and loading by extended culture, Arm C. Cryopreservation media one, CPM1, consisted of PLASMA-LYTE A (Baxter part 2B2544X) containing 6.8% m/v trehalose (Sigma part T0167), 2% hetastarch (Phizer part 00409-7248-03), 5% human serum

albumin (HSA) (Octapharma NDC 68209-643-02), and was used to directly resuspend cells post centrifugation. Cryopreservation media two, CPM2, was a three-part solution, where two parts cell suspension were combined with one part trehalose solution and 2 parts polyvinylpyrrolidone (PVP) solution (Sigma part PBP40). The PVP solution consisted of PLASMA-LYTE A supplemented with 30% m/v PVP. The trehalose solution consisted of PLASMA-LYTE A supplemented with 7.6% m/v trehalose. The control cryopreservation media was PLASMA-LYTE A with 10% v/v DMSO (Mylan part 67457-178-50) and 5% v/v HSA. For each condition formulated cell suspension was vialed 1 mL/vial. For each experimental condition, half of the cell suspension from each condition was filled into plastic Corning 2.0 mL vials (part 430659CE), and half filled into glass vials suitable for lyophilization. Plastic vials were placed in a Biocision cool cell (model FTS30 or LX) and frozen in a -80°C freezer. Glass vials were frozen in a Vertis lyophilizer (model genesis 25XL). The freezing profile used for the lyophilizer was to hold 8.0°C for 10 minutes, ramp to -60°C in 68 minutes, and hold -60°C until samples were removed and stored in a -80°C freezer. All freezing was carried out a pressure of 750 torr, near atmospheric pressure of 760 torr. Control cells from Arm were cultured in parallel, and formulated in control cryopreservation media. The control formulation was divided into plastic Corning 2.0 mL vials, and glass vials, 1 mL/vial, and frozen in a cool cell or the lyophilizer respectively along with vials from the experimental arm. All frozen samples were maintained at -80°C for a minimum of 14 days prior to thawing and analysis.

Arm A: ATP Mediated Trehalose Loading

Cells were seeded into 6 flasks total, 2 flasks as controls and 4 flasks as experimental conditions, Figure 1. Each experimental flask was subjected to trehalose loading at a different time point prior to harvest. One flask was loaded 24 hours prior to harvest, one flask was loaded

3 hours prior to harvest, one flask was loaded 90 minutes prior to harvest, and one flask was loaded 15 minutes prior to harvest. ATP mediated loading media was prepared for each flask by supplementing 25 mL of complete growth media with 2 grams of trehalose and 0.07 grams of ATP (Sigma part A6419), and filtering through a 0.2 μm filter (Millipore part 7850). Loading was accomplished by removing the spent media and adding 25 mL of ATP mediated loading media until harvest.

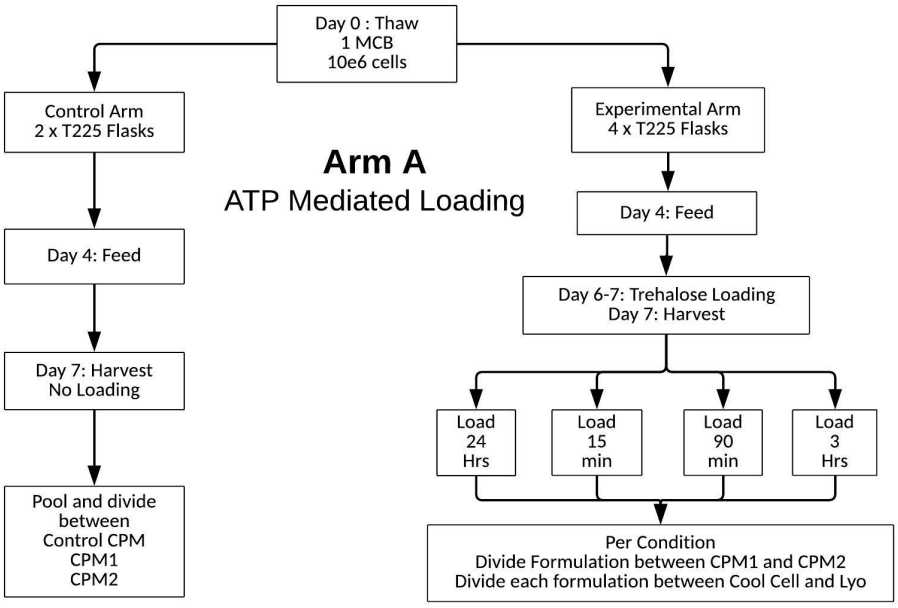


Figure 1. Experiment #1 Arm A process flow of ATP mediated trehalose loading. Four experimental arms were tested where cells were exposed to growth media containing ATP and high concentrations of trehalose for 15 minutes, 90 minutes, 3 hours, and 24 hours. A control condition was also cultured in parallel and cells from each condition were divided equally and frozen in CPM1 and CPM2.

Arm B: Loading by Spontaneous Pore Formation.

Cells were seeded into 6 flasks total, 2 flasks as controls and 4 flasks as experimental conditions, Figure 2. On the day of harvest one experimental flask was discarded and the remaining three flasks were subjected to trehalose loading at a different time point prior to harvest. One flask was loaded 90 minutes prior to harvest, one flask was loaded 60 minutes prior

to harvest, and one flask was loaded 30 minutes prior to harvest. Loading media was prepared, per flask, by combining 15 mL of PLASMA-LYTE A with 5 mL of 25% HSA, 5 mL of DMSO, 2 g of trehalose, and sterile filtering. Loading was accomplished by removing the spent media and adding 25 mL of DMSO loading media until harvest.

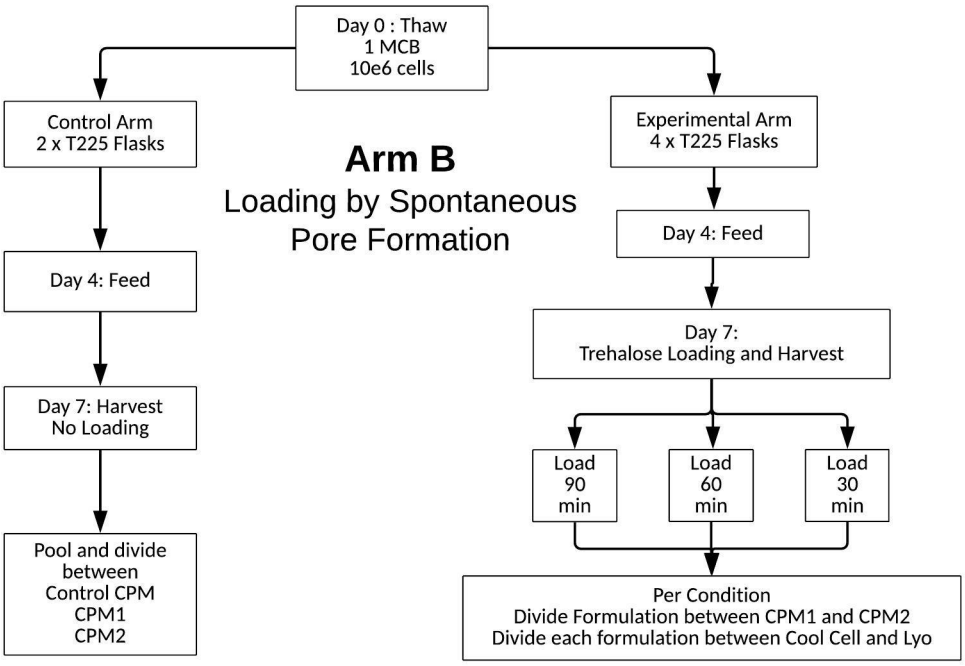


Figure 2. Experiment #1 Arm B process flow of trehalose loading by spontaneous pore formation. Three experimental conditions were tested where the cells were exposed to growth media containing 20% DMSO and high concentrations of trehalose for 30, 60, or 90 minutes. Cells from the control arm were pooled and divided equally into formulations of control cryopreservation media, CPM1 and CPM2. Each condition of the experimental arm was divided equally between formulations of CPM1 and CPM2. The subsequent vials of each condition were then divided equally among freezing in a cool cell or in lyophilizer.

Arm C: Loading by extended culture.

Cells were seeded into 6 flasks total, 2 flasks as controls and 4 flasks as experimental conditions, Figure 3. Two days prior to harvest two flasks were fed with loading media. Twenty-four hours later two additional flasks were fed with loading media, and 24 hours after that all vessels in Arm C were harvested. Loading media was prepared at the time of use by adding trehalose to complete growth media at a concentration of 0.04 g.mL. The loading media was then filtered through a 0.2 μm filter.

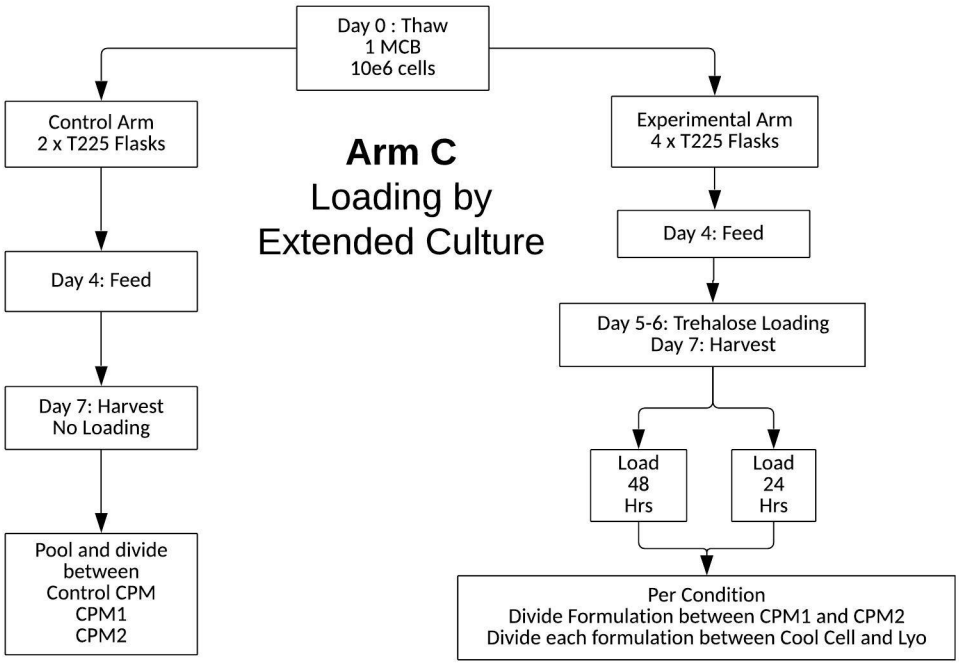


Figure 3. Experiment #1 Arm C process flow of trehalose loading by extended culture. Two experimental conditions were tested where cells were exposed to growth media containing high concentrations of trehalose for 24 and 48 hours respectively. Cells from the control arm were pooled and divided equally into formulations of control cryopreservation media, CPM1 and CPM2. Each condition of the experimental arm was divided equally between formulations of CPM1 and CPM2. The subsequent vials of each condition were then divided equally among freezing in a cool cell or in lyophilizer.

Cells from every arm and condition were thawed and analyzed on a NC-200 for viability. Cells formulated in control media from Arm A that were frozen in a cool cell, as well as cells from Arm C that were frozen in a lyophilizer were also thawed and analyzed on a NC-200. In all

cases cells were thawed in a plasmatherm at 37.0°C until only a small ice crystal remained. Cell suspension was then removed from the vial and resuspended in complete growth media targeting 500,000 to 1 million cells per mL based on the estimated number of cells in each vial. At this point all experimental loading methods, conditions, and formulations appeared to perform nearly as well, or better than the control formulations and conditions in terms of viability. It was then decided to utilize the cells that had been frozen in glass vials for further lyophilization. At least one vial from each condition remained at this point. The hope was that after lyophilization and rehydration there would be more disparity in the results of viability testing. Then one loading method and CPM could be chosen for further evaluation.

Initial lyophilization

The lyophilizer was programed to start at the coldest temperature the condenser could reach, -60°C, and the following profile. Hold for 10 min at -60°C and 750 torr; ramp for 60 minutes to -60°C and 450 torr; ramp for 63 minutes to -35°C and 80 torr; hold 2160 minutes at -35°C and 80 torr, ramp 275 min to 20°C and 80 torr, hold 360 min at 20°C and 80 torr, ramp for 60 min to 20°C and 300 torr, then end the program by capping the vials and returning the chamber to atmospheric pressure. This profile was chosen as it closely mimics what was performed in a similar study on hematopoietic stem and progenitor cells (Buchanan *et al.* 2010). An initial test run of the lyophilizer was carried out, and the profile appeared to execute as expected. For the first attempt, only two vials from Arm C were used, one vial formulated in CPM1 from the 24 hr loading time point, and one vial formulated in CPM2 from the 24 hr loading time point. The data logging software failed for this run, but the run completed in the estimated time and all steps appeared to execute as expected. The cell suspension in the vials

appeared to be in the form of a solid dry cake, and was not collapsed. The vials were then transferred to 2-8°C storage for approximately one week. They were then rehydrated and counted. Rehydration was performed as follows, add 1 mL of WFI water, wait 30 seconds, pipette up and down 5 times, add 2 mL of complete growth media drop wise, pipette up and down 5 times. One Corning T-25 and one Nunc T-25 flask were seeded at 1500 cells/cm^2 from each condition, but after overnight incubation at 37.0°C all flasks exhibited excessive bacterial growth and were discarded. At this point, several vials from each arm remained at this point, but in a few cases there were insufficient vials for a specific time point due to prior use or breakage. All of the remaining glass vials were placed into dry ice and their stoppers were replaced with vented stoppers suitable for lyophilization. The lyophilizer was chilled to -60°C before introducing the pre-frozen samples, and then the previously executed profile was initiated. Unexpectedly, it took more than 275 minutes to ramp from -35°C to 20°C. This was likely due to an excessively cold atmosphere in the room at the time. It is possible the initial run with only two vials from Arm C had a similar time variance, but was not captured. All vials exhibited the same dry cake appearance after lyophilization and none of the cakes were collapsed. Upon completion of the run all vials were stored in a refrigerator at 2-8°C.

Experiment 1: Rehydration Methods

Two methods of rehydration were evaluated on the lyophilized sample from experiment #1. The first method, or rehydration method #1, used sterile water for injection (WFI), and was carried out as follows, add 1 mL of WFI, wait 30 seconds, slowly pipette up and down at least 5 times, add 2 mL of complete growth media drop wise, slowly pipette up and down at least 5 times. The second method, or rehydration method #2, used only complete growth media, and

was carried out as follows, add 1 mL of complete growth media, wait 30 seconds, slowly pipette up and down at least 5 times, add 2 mL of complete growth media drop wise, slowly pipette up and down at least 5 times.

Experiment 1: Initial Rehydration

The first attempt to rehydrate cells was conducted three days post lyophilization, using rehydration method #1, on Arm C, 24 hour loading by extended culture, in CPM1. Cell from this rehydration were seeded into a Corning T-75 flask for 24 hours, but no results were obtained due to excessive bacterial growth. The glass vials used for lyophilization were not pre-sterilized and it is likely the added manipulation and necessity to lyophilize vials while open to a non-sterile atmosphere lead to contamination.

Experiment 1: Second Rehydration

The second rehydration was carried out between 22 and 24 days from lyophilization. This round of rehydration aimed to determine which rehydration method; method #1, using WFI; or method #2, using only complete growth media, would produce favorable results. Cells from Arm C, loaded for 24 hours by extended culture, lyophilized in CMP1 and CMP2 were rehydrated using both rehydration method #1 and method #2.

Experiment 1: Third Rehydration

The third round of rehydration aimed to evaluate the long-term stability of lyophilized cellular material among all of the arms with respect to initial viability. Rehydration of samples from all arms and as many conditions that remained was performed from 653 and 654 days post

lyophilization, or approximately one year and nine months from the time of lyophilization and storage at 2-8°C. Initially Arm C, 48 hrs loading by extended culture, was rehydrated using both method #1 and method #2, but all remaining samples were rehydrated using method #2. From these results, as discussed below, Arm C, 24 hrs loading, preserved in CPM1 was chosen for further evaluation.

Experiment 1: Forth Rehydration and Seeding

The final sample from this experiment was rehydrated 697 days post lyophilization. This sample was from Arm C, 24 hour loading by extended culture, in CPM2, and rehydration method #2 was utilized. This sample was chosen because it was the most similar to Arm C, 24 hour loading, preserved in CPM1, but all vials from that condition had been consumed. Recovered cells were diluted in complete growth media containing 0.2% GA-1000 (Lonza part CC-4083), an antibiotic and antifungal agent suitable for extended cell culture, then seeded into one Corning T-225 flask at 1100 cells/cm^2. The culture was monitored for 72 hours, but the cells did not attach to the surface or appear to proliferate in anyway and the flask was discarded.

Experiment #2: Verification of Loading by Extended Culture and Lyophilization using CMP1

The goals of this experiment were to repeat the results from loading by extended culture for 24 hours and lyophilizing using CPM1 from experiment #1. A number of controls were added to this experiment to evaluate the effects of loading when frozen in a controlled rate freezer (CRF), Thermo CryoMed model 7452, or completely lyophilized, as well as the use of CPM1 as a cryopreservation media alone when frozen in a CRF or completely lyophilized, and a

negative control was included to evaluate the effect of lyophilization on cells preserved in the control DMSO based cryopreservation media without exposure to trehalose loading or CPM1, Figure 4. All glass vials and stoppers used for lyophilization were pre-sterilized in an autoclave prior to use, and all manipulation were carried out in a biological safety cabinet. The process flow diagram, Figure 4, below details the experimental scale and timeline, as well as the conditions of each arm.

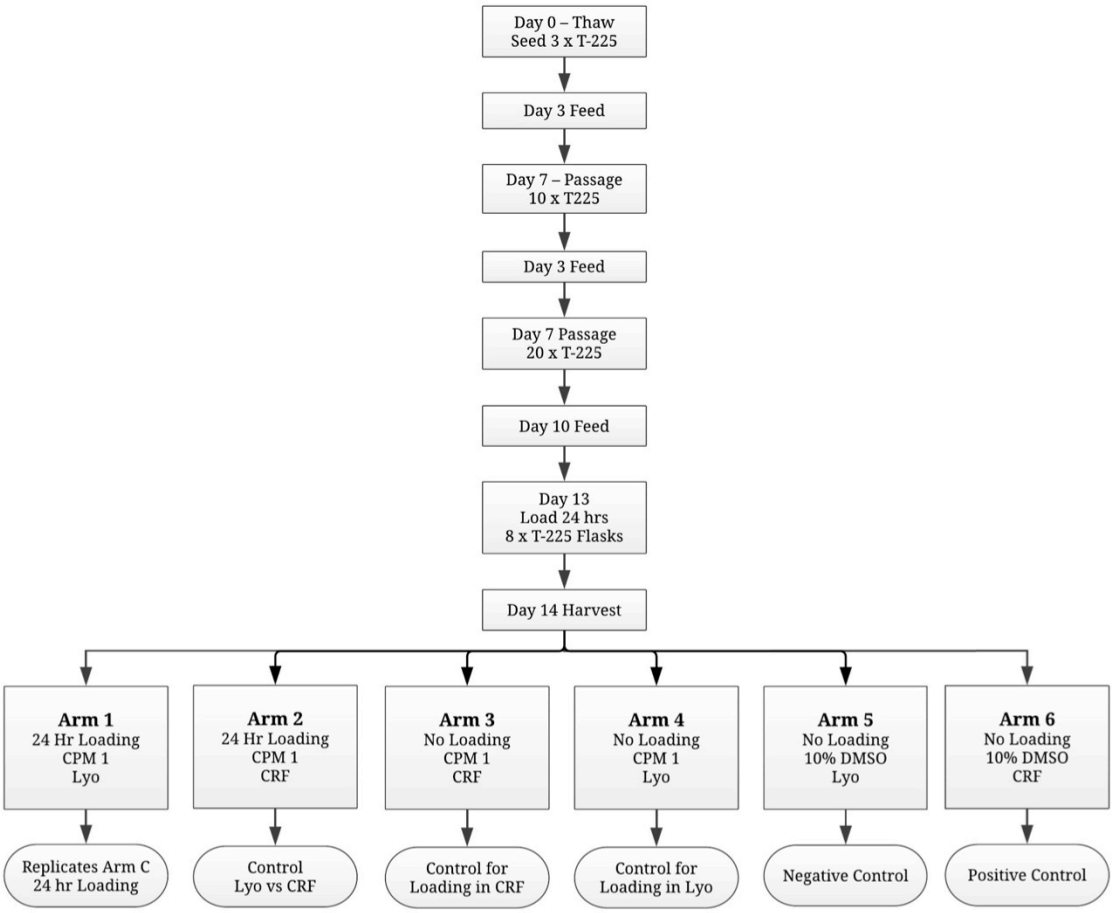


Figure 4. Experiment #2 process flow diagram. After the seeding and expansion of hMSCs five arms were generated. Arm 1, experimental condition, consisted of cells being exposed to trehalose loading media for approximately 24 hours prior to harvest and formulation with CPM1 followed by lyophilization. Arm 2, control for lyophilization, consisted of cells exposed to trehalose loading media for approximately 24 hours prior to harvest and formulation with CMP1, and frozen in a CRF. Arm 3, control for trehalose loading and freezing in a CRF, consisted of cells formulated in CPM1 and frozen in a CRF without being exposed to loading media. Arm 4,

a control for trehalose loading and lyophilization, consisted of cells formulated in CMP1 and lyophilized without being exposed to loading media. Arm 5, a negative control, consisted of cells formulated in the control cryopreservation media and lyophilized without any exposure to loading media or CPM1. Arm 6, a positive control, consisted of cells formulated in the control cryopreservation media and frozen in a CRF without exposure to loading media or CPM1.

Cells were cultured using the conditions established in the preliminary cell culture evaluation but a passage was added to expand the cells into 20 flasks. For this experiment the final formulation was carried out slightly differently than in the previous experiment. After centrifugation, all arms were initially resuspended in half their final volume with PLASMA-LYTE A supplemented with 5% HSA. Arms 1, 2, 3, and 4 were the brought to their final volume use a 2X version of CPM1, consisting of PLASMA-LYTE A supplemented with 13.6% m/v trehalose, 4% hetastarch, and 5% HSA. This method of formulation should have allowed for more complete removal of any residual growth media while targeting the same formulation executed in Arm C of the previous experiment. After a total of 14 days in culture cells were exposed to five different conditions or procedures. Arm 1 and Arm 2 were both exposed to trehalose loading media on day 13, approximately 24 hours prior to harvest, and both of these arms were formulated in CPM1. Arm 1 was the lyophilized and stored at 2-8°C, while Arm 2 was frozen in a CRF and stored at -80°C. Arms 3, 4, and 5 were not exposed to trehalose loading media. Arms 3 and 4 were both formulated in CPM1. Arm 3 was then frozen in a CRF and stored at -80°C. Arm 4 was lyophilized and stored at 2-8°C. Arm 5 was formulated in control CPM, lyophilized, and stored at 2-8°C.

The lyophilization profile for this experiment was modified slightly from the previous experiment, Table 1. The final stages of warming were reduced to 15°C from the previously used 20°C. This was done so that if the room temperature was below 20°C the lyophilizer would still be able to warm to a sufficient temperature to complete the profile in a shorter amount of

time. The modified lyophilization profile is described in the table below. Step 5 was lengthened and step 6 was added, from the previous run, to allow additional time for warming. At the completion of the run the lyophilized cell suspension had the appearance of a dry cake and was not collapsed.

Table 1. Lyophilization profile for Experiment #2.

Thermal Treatment (all steps at pressure of 750 torr)	
Step 1	Hold at 8°C for 10 minutes
Step 2	Ramp to -60°C for 68 minutes
Step 3	Hold at -60°C for 120 minutes
Primary Drying	
Step 1	Hold at -60°C and 750 torr for 10 minutes
Step 2	Ramp at -60°C to 450 torr for 60 minutes
Step 3	Ramp to -35°C and 80 torr for 63 minutes
Step 4	Hold at -35°C and 80 torr for 2160 minutes
Step 5	Ramp to 15°C and 80 torr for 330 minutes
Step 6	Ramp to 15°C and 80 torr for 20 minutes
Step 7	Hold at 15°C and 80 torr for 360 minutes
Step 8	Ramp at 15°C to 300 torr for 60 minutes
Post Hold at 20°C and 300 torr until end	

For the arms frozen in a CRF a profile was constructed to mimic the thermal treatment steps of the lyophilization profile. The freezing profile is detailed in Table 2 below. When the profile ended all samples were transferred to -80°C for storage.

Table 2. Controlled rate freezer profile used in Experiment #2.

Step 1	Wait at 10.0°C
Step 2	Wait at Chamber = 5.0°C until sample = 8.0°C
Step 3	Hold 8.0°C for 10 minutes
Step 4	Ramp 1.0°C/min until chamber = -60°C
Step 5	Hold -60°C for 120 minutes
Step 6	End

Experiment #2: Rehydration and Seeding

The first rehydration and thawing of samples from experiment #2 was carried out two weeks after cryopreservation, or 12 days after the completion of lyophilization. Rehydration was conducted using method 2 for all lyophilized samples. The sample from Arm 5 did not easily dissolve and required additional time and significant pipetting to reconstitute. Samples frozen in a CRF were thawed in a plasmatherm, as described in the preliminary cell culture evaluation. A small volume of cell suspension from each arm was analyzed on a NC-200 for viability. The remaining volume was centrifuged for 10 minutes at 500 x g and 5°C. The subsequent cell pellets were resuspended in complete growth media containing 50 mg/L gentamicin, Lonza part 17-519Z, and seeded into a minimum of two Corning T-75 flasks at 8000 cells/cm^2. All flasks were then incubated at 37.0°C in a humidified incubator. Approximately 24 hours later one flask from each arm was harvested to evaluate seeding efficiency. The supernatant was collected, centrifuged for 10 minutes at 500 x g and 5°C, resuspended in 0.5 mL of complete growth media and analyzed on a NC-200. Cells were collected from each flask by exposure to trypsin for 12 minutes and subsequently quenched with complete growth media. Each sample was then

centrifuged, resuspended, and analyzed on a NC-200 in the same manner as the supernatant samples. During exposure to trypsin it was observed that some of the clumps of material still present in the Arm 5 flask appeared to dissolve. Presumably the trypsin was able to dissociate cells from one another or from a substrate that many have been created during the lyophilization process. At this point Arm 5 was terminated due to lack of attachment and very poor viability. Flasks in Arm 1 and Arm 3 were cultured until day 8 with a feed on day 3, but ultimately discarded due to lack of any evidence of proliferation. Arm 2, Arm 4, and Arm 6 were all cultured up to day 13 with feeds on day 3 and day 8. Arm 6 appeared to reach confluence around day 7. Arm 2 and Arm 4 both reached approximately 90% confluence on day 13.

Samples from Arm 1, Arm 2, and Arm 6 were rehydrated or thawed and analyzed by fluorescence-activated cell sorting (FACS) for the presence of surface markers specific to this cell bank. The markers that were evaluated were CD45, CD73, HLA-DR, CD14, CD34, CD90, CD105, and CD19. Though the project did not have access to the specific reference cell banks, the cells grown in Arm 6 can be considered to be a reference control as they were expanded, harvested, and cryopreserved under normal conditions, and demonstrated normal growth and morphology after subsequent thaw and expansion.

RESULTS

Preliminary Cell Culture Evaluation

The method based approach of this project necessitated the establishment of evidence that the cells chosen and procedures used were adequate to produce a certain level of cell proliferation and viability. A baseline needed to be established using repeatable methods so that subsequent experiments could be conducted under the same conditions, with the exception of those procedures altered for experimental reasons. Thus a preliminary experiment was conducted to evaluate the potential growth profile and viability that could be expected in a control environment. After seven days of incubation, with one feed, the cell culture was greater than 95% confluent. The harvest of one T-225 yielded 10.6 million cells with a viability of 99.6%. This data served as confirmation that the cell bank, culture media, reagents, and procedures were adequate to support a reasonable level of proliferation and maintain a high level of viability. These procedures were then established as the control condition for all experiments.

Experiment #1

The first experiment conducted evaluated three different loading strategies for trehalose and two different DMSO-free cryopreservation medias with respect to their use in a ultralow temperature environment after cryopreservation as well as after lyophilization and storage in a warmer 2-8°C environment. Additionally, two different methods of rehydration were evaluated to reconstitute lyophilized cells.

The initial portion of the experiment was to evaluate the use of CPM1 and CPM2 as a cryopreservation media. Cells were cultured under the control conditions and formulated in CPM1, CPM2, or the control CPM containing 10% DMSO. Additionally, samples were frozen using a cool cell as well as using a lyophilizer. This was done to demonstrate the ability of the

lyophilizer to freeze cells with comparable results to a more traditional direct freezing system. Of all of the control cultures, the condition that exhibited the lowest viability post thaw was preservation in control media using a Cool Cell. This was the condition most similar to standard cell culture practice. Cells were not exposed to any experimental conditions. They were formulated in 10% DMSO and frozen at a target rate of approximately 1°C per minute. The viability of the control cells frozen in control CPM in a Cool Cell was 86.4%, Figure 5. That is slightly lower than expected, and lower than all other conditions. For reference, the viability at thaw for these cells during the preliminary cell culture evaluation was 95.0% and at the thaws for Experiment #1 it was 96.2% for Arm A, 95.0% for Arm B, and 95.4% for Arm C, Figure 5. From those counts the average expected viability of cells from this bank would be approximately 95.4%. The control condition was preserved in the same type of media as the original cell bank, and a similar viability was expected here. Even discounting the unexpectedly low viability of the control cells, all of the experimental conditions, including control cells frozen in a lyophilizer, met or exceeded the expected viability of these cells after cryopreservation.

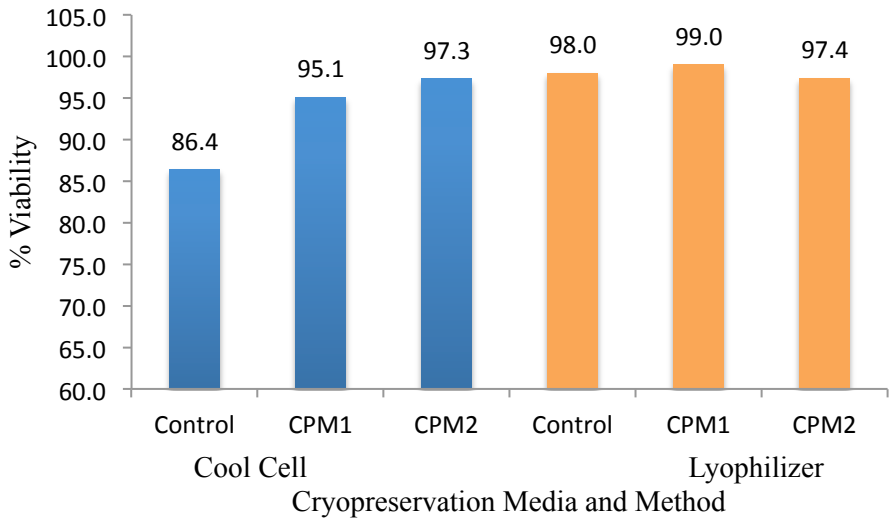


Figure 5. Viability of control cells after cryopreservation in CPM1, CPM2, or control media, and frozen in a Cool Cell or a lyophilizer. None of these cells were exposed to trehalose loading conditions. Cells were thawed as described in materials and methods then analyzed on a NC-200 for viability. The results from cells frozen in a cool cell are depicted on the left in blue, and the results from cells frozen in a lyophilizer are depicted on the right in orange.

Similar results were also obtained from Arm A cells exposed for various times to ATP mediated trehalose loading media, Figure 6. All conditions had viability greater than the 86.4% of the control cells frozen in a Cool Cell, and all were near the expected average viability of this cell bank post thaw. The lowest viability recovered from these conditions was 92.3% from the cells exposed to ATP mediated trehalose loading media for 24 hours, formulated in CPM2, and frozen in a lyophilizer. This trend was also mirrored in the cells frozen in a Cool Cell, where cells exposed to ATP mediated trehalose loading media for 24 hours also had the lowest viability. Additionally, of the cells formulated in CPM1 previously exposed to ATP mediated trehalose loading media for 24 hours had lower viability that similar conditions exposed for less time to ATP mediated trehalose loading media. There also appears to be a trend that cells frozen in CPM1 have slightly higher viability than cells exposed to the same conditions but formulated in CPM2. The only exception is the 15-minute time point for cells frozen in a cool cell. This could suggest that approximately 90 minutes of exposure to ATP mediated trehalose loading

media is optimal, and CPM1 is slightly more effective in most cases than CPM2, but none of these results could be considered a definitive failure considering they all out performed the control cells with respect to viability.

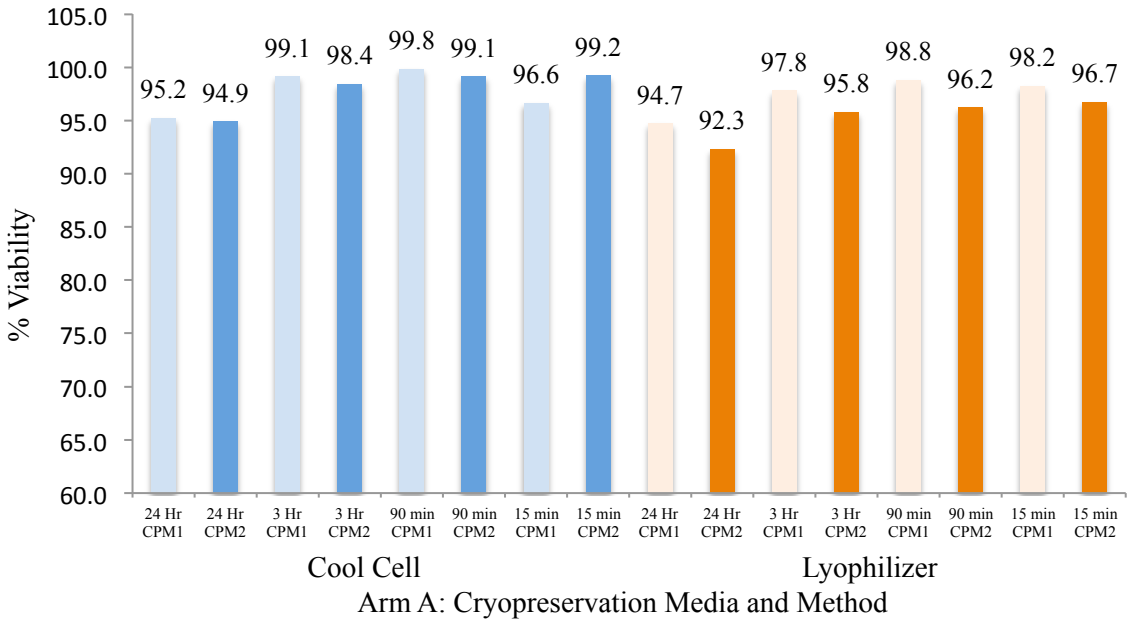


Figure 6. Experiment #1, Arm A, loading by ATP mediated trehalose loading. Cells were exposed to trehalose loading media for various amounts of time, harvested, formulated in either CPM1 or CPM2, and frozen in either a Cool Cell or a Lyophilizer. Those cells were then thawed and analyzed on a NC-200 to assess their viability.

The results from Arm B, cells exposed to DMSO mediated trehalose loading media had more variability, Figure 7. All of the conditions formulated in CPM1 and frozen in a Cool Cell had lower viabilities post thaw compared to all of the other conditions. Of the 30 minute, 60 minute, and 90 minute time points for cells preserved in CPM1, their viability post that was 83.0%, 83.0%, and 78.4% respectively. This slight drop in viability over time with cells cryopreserved in CPM1 and frozen in a cool cell is not observed in cells cryopreserved in CPM2 or frozen in a lyophilizer. All of the other conditions exhibited viabilities between 96.8% and 99.6%, higher than the 86.4% observed in the control cells or the expected approximate 95.4% from this cell bank. At this point, even though CPM1 performed poorly when used in

conjunction with DMSO mediated trehalose loading and freezing in a cool cell, it could not be completely ruled out as a possibility for use as a lyophilization technique.

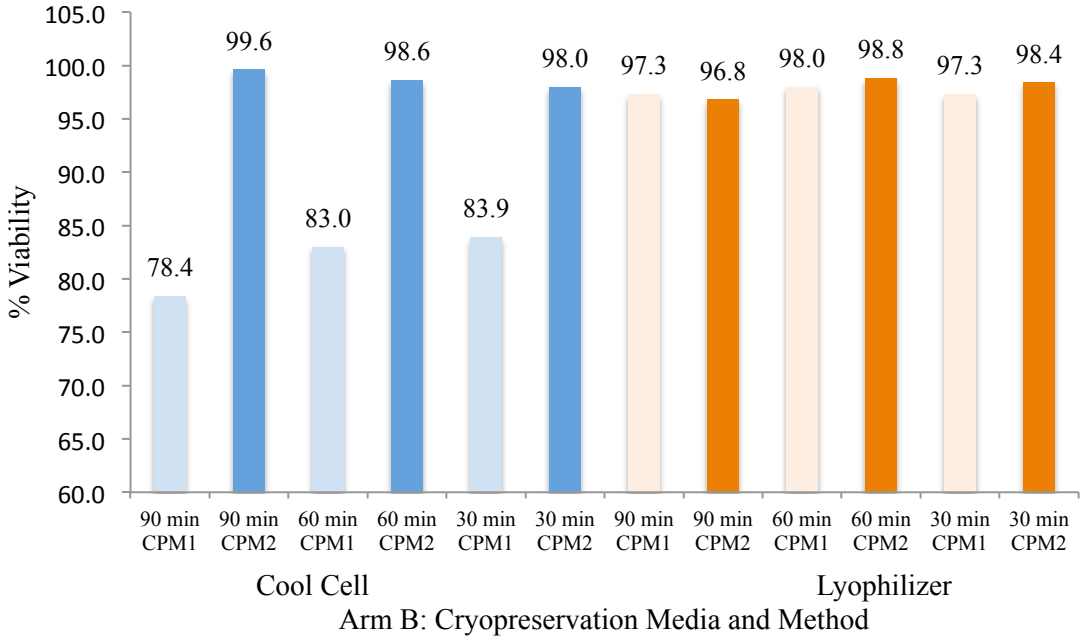


Figure 7. Experiment #1, Arm B, Loading by spontaneous pore formation. Cells were exposed to DMSO mediated trehalose loading media for various amounts of time, harvested, formulated in either CPM1 or CPM2, and frozen in either a Cool Cell or a Lyophilizer. Those cells were then thawed and analyzed on a NC-200 to assess their viability.

Arm C, loading by extended culture, also performed as well or better than the control cells with respect to viability. The lowest viability observed from these conditions was 96.8% from the cells exposed to growth media concentrated with trehalose for 24 hours and formulated in CPM2, Figure 8. Considering all other viabilities were between 97.4% and 99.0%, none of the experimental conditions could be considered to have a negative effect.

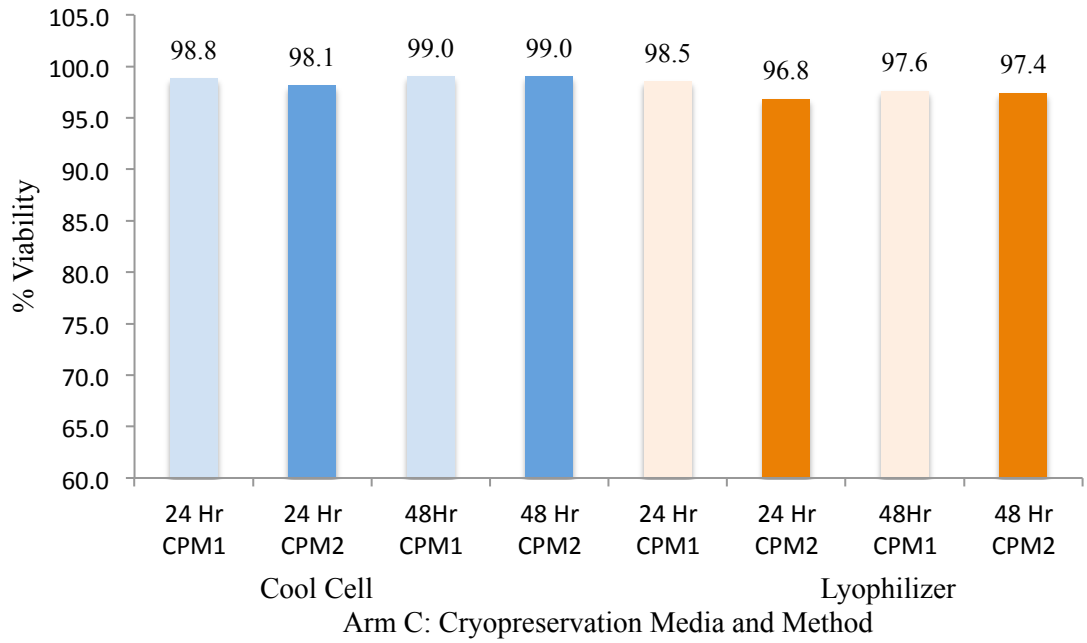


Figure 8. Experiment #1, Arm C, Loading by extended culture. Cells were exposed to complete growth media supplemented with trehalose and allowed to incubate for 24 or 48 hours. They were then harvested, formulated in either CPM1 or CPM2, and frozen in either a Cool Cell or a Lyophilizer. Those cells were then thawed and analyzed on a NC-200 to assess their viability.

The apparent success when thawing all of conditions from experiment #1 lead to the decision to further lyophilize the remaining samples formulated in glass vials. From those lyophilized samples, several attempts to rehydrate them were made, and the viability of those samples was again analyzed on a NC-200. The initial attempt, as described in the materials and methods section, was on cells from Arm C, 24 hour loading by extended culture that were cryopreserved and lyophilized in CPM1. The initial viability observed was 65.2%, which slowly fell over a period of three and a half hours to 30.9%, Figure 9.

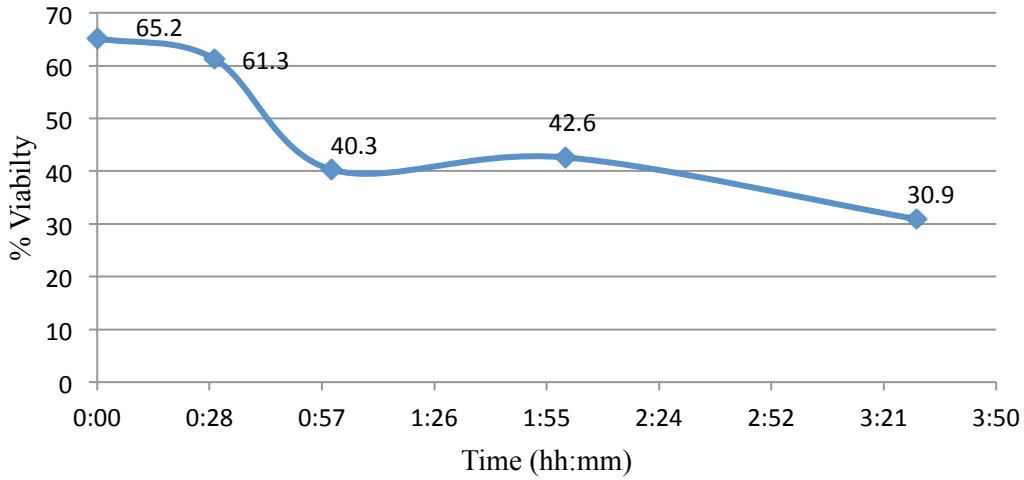


Figure 9. Rehydration of cells lyophilized in CPM1 six days post lyophilization. This was the initial rehydration of cells from Arm C, 24 hour loading, that were cryopreserved and lyophilized in CPM1. These cells were rehydrated using method 1 and their viability was assessed over 3.5 hours using a NC-200.

The second attempt at rehydration evaluated two different methods for hydrating cells, and both methods were used on cells lyophilized in CPM1 and cells lyophilized in CPM2. As described in materials and methods, Method 1 used water to initially hydrate the cells followed by dilution in complete growth media, whereas Method 2 only used complete growth media. At the initial time point of rehydration the cells lyophilized in CPM1, rehydrated using method 1, exhibited a viability of 67.5% and the cells rehydrated using method 2 exhibited a viability of 77.1%. Over the course of 280 minutes of storage at room temperature the cells were analyzed by NC-200 eight times, and had an average viability of 69.8%. In parallel, the cells rehydrated in method 2 had an average viability of 73.0%, Figure 10.

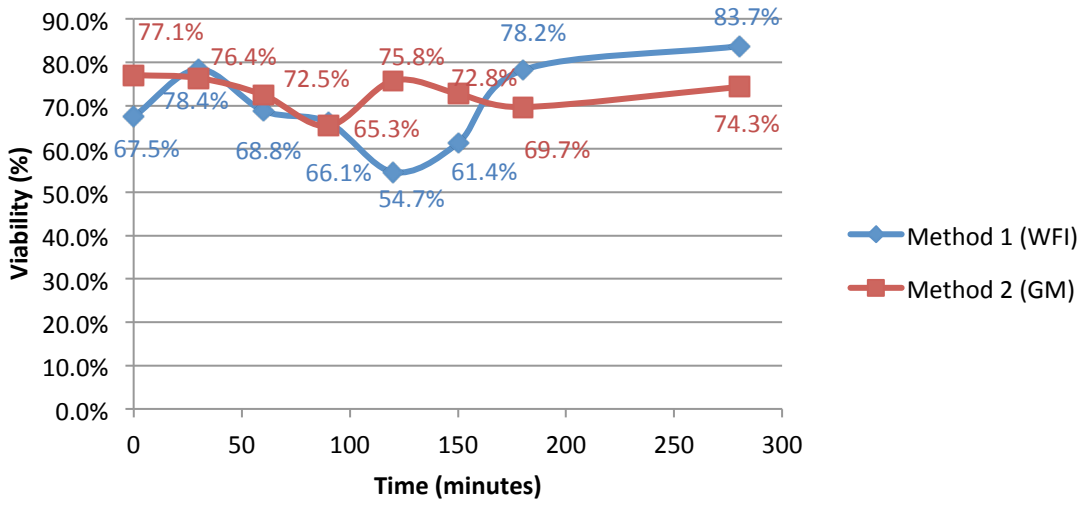


Figure 10. The rehydration of cells lyophilized in CPM1 22 days post lyophilization. This was the second rehydration of cells from Arm C, 24 hour loading, that were cryopreserved and lyophilized in CPM1. These cells were rehydrated using Method 1 as well as Method 2. Their viability was assessed over 3.5 hours using a NC-200.

Similar results were obtained when cells from Arm C, 24 hour loading, lyophilized in CPM2 were rehydrated using Method 1 and Method 2. At the initial time point of rehydration of cells lyophilized in CPM2 the cells rehydrated using method 1 exhibited a viability of 59.3% and the cells rehydrated using method 2 exhibited a viability of 63.7%. Over the course of 280 minutes of storage at room temperature the cells rehydrated using Method 1 had an average viability of 54.2% and the cells rehydrated in Method 2 had an average viability of 64.8%, Figure 11.

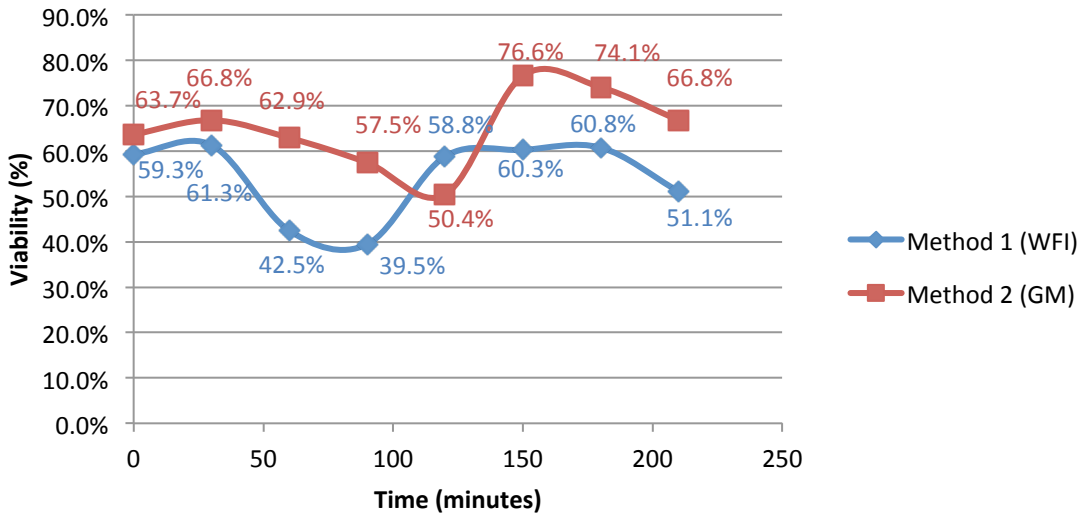


Figure 11. The rehydration of cells lyophilized in CPM2 24 days post lyophilization. This was the first rehydration of cells from Arm C, 24 hour loading, that were cryopreserved and lyophilized in CPM2. These cells were rehydrated using method 1 as well as method 2. Their viability was assessed over 3.5 hours using a NC-200.

The third round of rehydrating was conducted on Arm C, 48 hrs; Arm A, 15 min; Arm A, 90 min; Arm A, 3 hrs; Arm B, 30 min; Arm B, 60 min; Arm B, 90min. Only samples of Arm C formulated in CPM2 remained, where as all other remaining samples from Arms A and B were formulated in CPM1. Initially samples from Arm C, 48 hr loading, were hydrated using both method 1 and method 2. The sample hydrated using method 1 had an initial average viability of 53.9% and the sample hydrated using method 2 had an average viability of 55.0%. All remaining samples were rehydrated using method 2, and each sample was analyzed on a NC-200 four times, Figure 12. Of the remaining experimental samples hydrated, Arm C, 48 hrs loading, was the highest recorded viability. In Arm A the highest viability recorded was 35.3% and in Arm B the highest recorded viability was 20.1%.

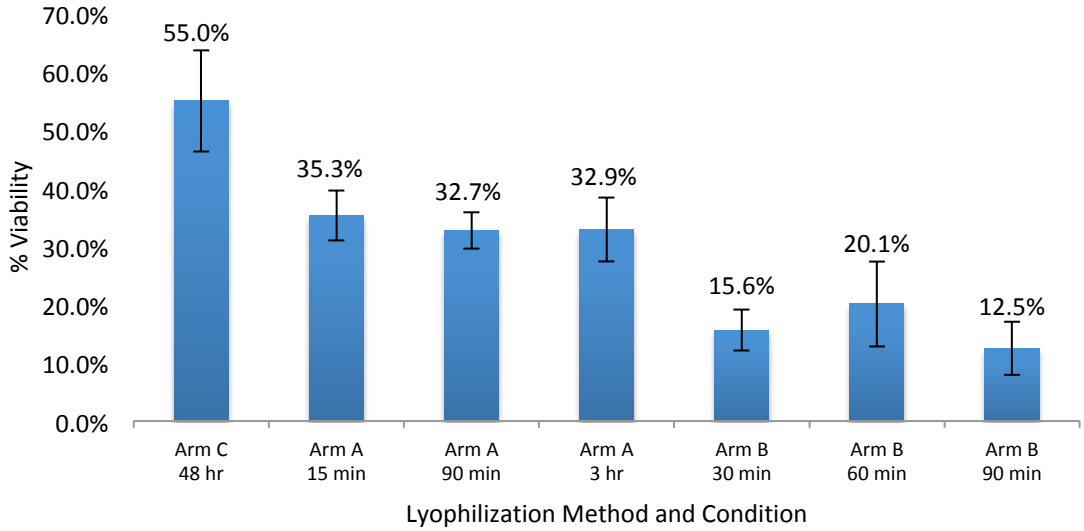


Figure 12. The rehydration of cells, 653 days post lyophilization in Experiment #1. All samples were rehydrated using method 2, and except for Arm C all samples had been lyophilized in CPM1. Viability was assessed using a NC-200 immediately after rehydration.

A complete set of control samples were also rehydrated using method 2 at this time. For each experimental arm, a control condition was cultured and processed alongside the experimental conditions. In all cases, regardless of the Arm letter designation, these control conditions were treated the same in terms of culture schedule, feeds, incubation conditions, and harvest conditions. No control conditions were exposed to trehalose loading. At the final harvest cell suspension from each control condition was divided equally among three formulations, control, CPM1, and CPM2. As a result, there were triplicate samples of cells exposed to the same formulation conditions for analysis at rehydration, Figure 13.

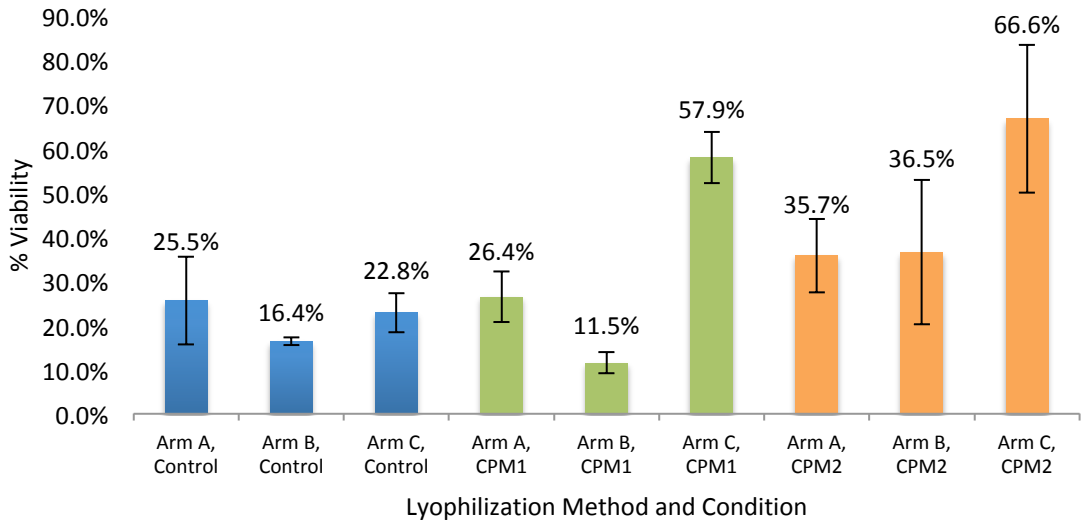


Figure 13. The rehydration of cells, 653 days post lyophilization, from the control arms of Experiment #1. All samples were rehydrated using method 2. In each arm control cells were formulated with three different cryopreservation media, but otherwise treated the same. Viability was assessed using a NC-200 immediately after rehydration.

The highest recorded average viability of 66.6% was observed in the control cells formulated in CPM2 from Arm C. This data point also had the highest standard deviation among individual samples at 16.7%, Table 3. However, the greatest variability was observed when data from all of the control samples formulated in the same cryopreservation media was averaged together. The standard deviation of all control samples analyzed in CPM2 was 20.3%, and control samples formulated in control media had a standard deviation of 18.9%, both of which appear more variable than any of the experimental conditions. The increased variability in data from the control conditions could be a result of several samples being generated from different arms at different times, and just representative of the inherent variability in processing. A more detailed statistical analysis of the variability in data from the experimental arms is not possible because the experiment could not be repeated in the way the control conditions were replicated in each arm.

Table 3. Average viability and standard deviation from the third rehydration of samples generated in Experiment #1. Each sample was analyzed four times on a NC-200 to calculate the average viability and standard deviation.

Condition	Average Viability	Standard Deviation
Arm C, 48 hr, CPM2	55.0%	8.7%
Arm A, 15 min, CPM1	35.3%	4.3%
Arm A , 90 min, CPM1	32.7%	3.1%
Arm A, 3 hr, CPM1	32.9%	5.5%
Arm B, 30 min, CPM1	15.6%	3.5%
Arm B, 60 min, CPM1	20.1%	7.3%
Arm B, 90 min, CPM1	12.5%	4.6%
Arm A, Control, Control CPM	25.5%	9.9%
Arm B, Control, Control CPM	16.4%	0.9%
Arm C, Control, Control CPM	22.8%	5.8%
Arm A, Control, CPM1	26.4%	5.7%
Arm B, Control, CPM1	11.5%	2.4%
Arm C, Control, CPM1	57.9%	4.4%
Arm A, Control, CPM2	35.7%	8.3%
Arm B, Control, CPM2	36.5%	16.3%
Arm C, Control, CPM2	66.6%	16.7%

Table 4. The average viability after rehydration of each control arm, by type of CPM used, in Experiment #2. Each data point was calculated using the four individual NC-200 counts from each control condition of each of the three arms in experiment #1, giving a total of twelve data points for each control condition.

Control Condition Formulation Media	Average Viability	Standard Deviation
Control CPM	33.2%	18.9%
CPM1	20.2%	8.0%
CPM2	46.3%	20.3%

Experiment #2

The second experiment focused on replicating the results of Arm C, 24 hour loading, using CPM1, Figures 9, 10 and 13. A number of control conditions were also added to specifically evaluate lyophilization, trehalose loading, and the use CPM1 as a DMSO-free

cryopreservation medium, Figure 14. At harvest the average yield was $12.0x10^6$ viable cells per flask with a viability of 97.4%. When initially rehydrated or thawed, the sample with the highest viability was Arm 6, no loading preserved in control cryopreservation media and frozen in a CRF, at 95.8%. The sample with the lowest viability was Arm 5, no loading preserved in control cryopreservation media and lyophilized, at 18.4%. This was expected as Arm 6 was a positive control and Arm 5 was a negative control. Of the four experimental arms, both Arm 2 and 4 that were frozen in a CRF exhibited higher viability than the corresponding Arms 1 and 3 that were lyophilized, 93.1% and 88.8% versus 74.1% and 66.0% respectively.

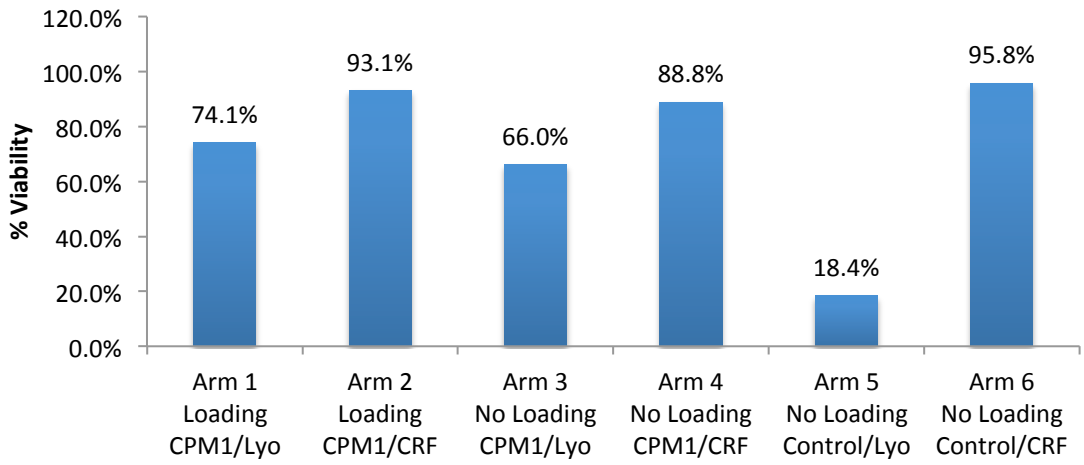


Figure 14. Experiment #2 viability results after thaw or rehydration. Arm 1 represents the complete experimental condition, trehalose loading, CPM1, and lyophilized. Arm 3 is the other experimental arm, but was not exposed to trehalose loading. Arm 2 was an experimental arm, but frozen in a CRF. Arm 4 was experimental, but not exposed to trehalose loading and was frozen in a CRF. Arm 5 was a negative control, not exposed to trehalose loading, formulated in control cryopreservation media, and lyophilized. Arm 6 was a positive control, not exposed to trehalose loading, formulated in control cryopreservation media, and frozen in a CRF.

When cells from each arm were seeded into T-225 flasks and their growth observed over thirteen days, evidence of damage during the lyophilization process became more apparent. Arm 5, the negative control using the control cryopreservation media and lyophilized did not dissolve well during rehydration. The lyophilized cellular material appeared to break up in to clumps, but

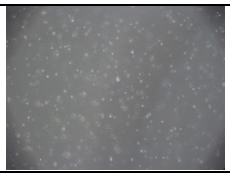
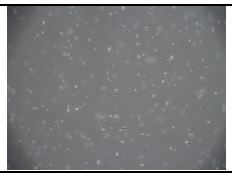
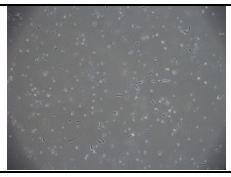
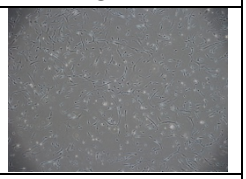
did not completely dissolve. After one day of culture the clumps remained, cells did not appear to be attached, and the cells that were recovered exhibited very low viability, thus Arm 5 was terminated after one day of culture, Table 5. The remaining arms that underwent lyophilization, Arm 1 and 3, failed to proliferate, Table 6. Though both of these arms appeared to have sufficient viable cells, those cells never appeared to attach to the surface of the flasks. When one flask from each condition was harvested 24 hours after seeding several observations were made. When the spent media was removed from the Arm 5 flask, some clumps of cells remained stuck to the flask. When exposed to trypsin these clumps appeared to break up. This likely led to an artificially high cell count of the harvested cells for Arm 5. Additionally when the spent media of Arm 5 was centrifuged and resuspended the cell clumps appeared to break up considerably, this likely caused the considerably higher total cell count in the Arm 5 spent media compared to the other arms. All of the lyophilized conditions had lower viability than the CRF conditions. All of the CRF conditions exhibited a higher percentage of the cells seeded in the harvested flasks than the lyophilized conditions. Arm 5 was an exception, but is artificially high due to unattached cell clumps remaining in the flask.

Table 5. Seeding efficiency of each arm in Experiment #2. One flask from each condition in Experiment #2 was harvested after 24 hour of incubation. Cells were collected by trypsinization as well as from the spent media. After concentration by centrifugation and resuspension in complete growth media each sample was analyzed on a NC-200. Data from Arm 5 is likely not accurate due to clumps of unattached cells that could not be completely removed prior to harvest.

Condition	TVC Seeded	Seeding Efficiency of Harvested Cells			Seeding Efficiency of Cells in Spent Media		
		Total Cells	% of Cells Seeded	Viability	Total Cells	% of Cells Seeded	Viability
Arm 1, Loading, CPM1, Lyo	6.00E+05	3.78E+03	0.6%	81.9%	5.70E+05	95.0%	20.5%
Arm 2, Loading, CPM1, CRF	6.00E+05	5.20E+04	8.7%	92.4%	3.27E+05	54.4%	63.6%
Arm 3, No Loading, CPM1, Lyo	6.00E+05	3.61E+03	0.6%	48.6%	4.46E+05	74.3%	31.5%
Arm 4, No Loading, CPM1, CRF	6.00E+05	4.63E+04	7.7%	85.3%	3.04E+05	50.6%	70.6%
Arm 5, No Loading, Control CPM, Lyo	4.13E+05	2.03E+05	49.0%	27.4%	1.78E+06	429.8%	59.7%
Arm 6, No Loading, Control CPM, CRF	6.00E+05	3.10E+05	51.6%	93.4%	3.22E+05	53.6%	84.1%

The subsequent culture and confluence tracking closely mirrored the seeding efficiency data. The arms from lyophilized cells, Arm 1 and 3, had the lowest percentage of cells attached, and did not proliferate. Arms 2 and 4 had a higher percentage of attachment, and did eventually grow to confluence, but their growth lagged in comparison to Arm 6. Arm 6, the control condition, exhibited the highest attachment after 24 hours and also reached confluence several days earlier.

Table 6. Experiment #2 confluence tracking of cells seeded post lyophilization or cryopreservation.

Day	Arm 1 Loading, CPM1, Lyophilized	Arm 2 Loading, CPM1, CRF	Arm 3 No Loading, CPM1, Lyophilized	Arm 4 No Loading, CPM1, CRF	Arm 6 No Loading, Control CPM, CRF
3					
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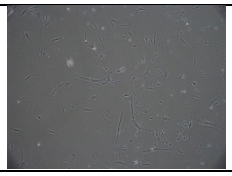
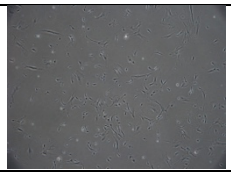
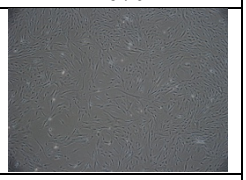
6					
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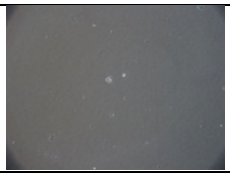
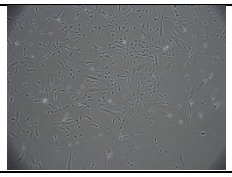
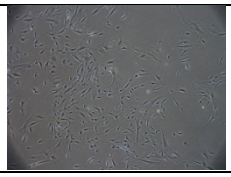
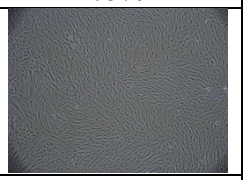
8					
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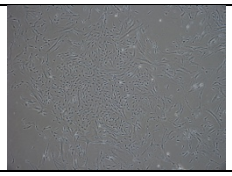
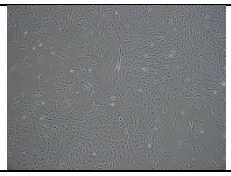
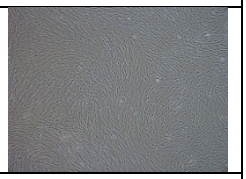
10	Terminated		Terminated		
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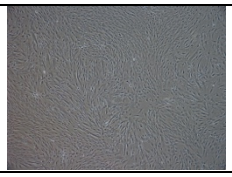
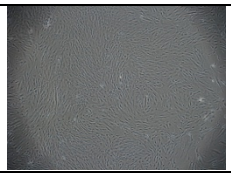
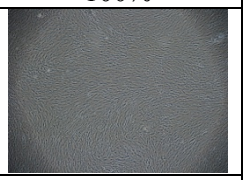
13	Terminated		Terminated		
	n/a	90%	n/a	90%	100%

Cells from Arms 1, Arm 2, and Arm 6 were analyzed for the presence or absence of CD45, CD73, HLA-DR, CD14, CD34, CD90, CD105, and CD19 using Flow cytometry, Figure 15. Arm 2 and Arm 6 exhibited very similar surface marker profiles, the signals were clear from the background and a sufficient number of events were captured to have a high confidence in the data. Arm 1 appears to be positive CD73, HLA-DR, CD14, CD90, and CD105, but the number of events captured is very low, and could be highly influenced by the background, Table 7. It is not possible from this data to confirm the presence of any specific surface markers and cells from Arm 1.

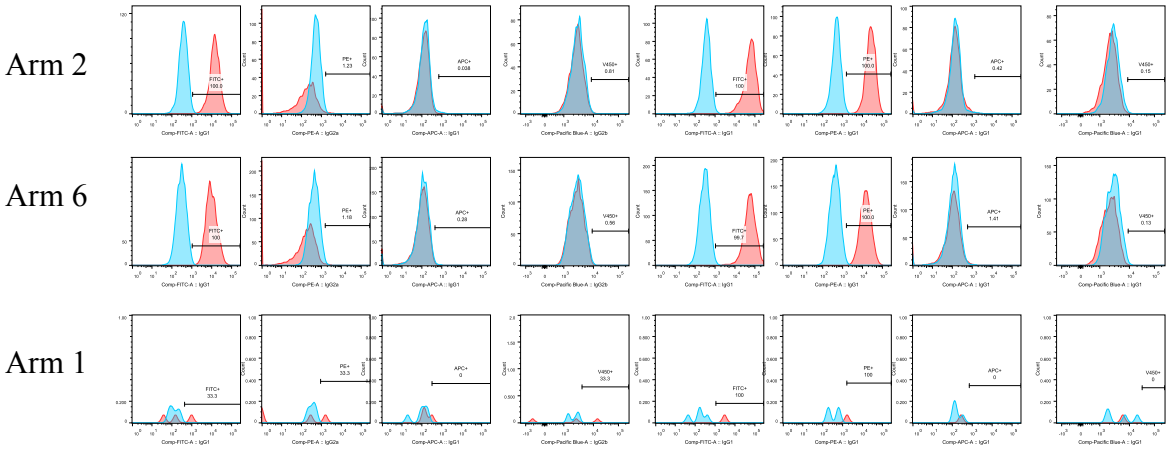


Figure 15. Raw data of FACS results from Arm 2, Arm 6, and Arm 1. Cells in Arm 2 had been exposed to trehalose loading and cryopreserved in CPM1 using a CRF. Cell in Arm 6, the control condition, were not exposed to trehalose loading and were cryopreserved in the control cryopreservation media. Cell in Arm 1 were exposed to trehalose loading and lyophilized in CPM1.

Table 7. Analysis of FACS data on Arm 2, Arm 6, and Arm 1. The values expressed in this table represent the percentage of the cells analyzed that expressed each surface marker.

Arm	CD45	CD73	HLA-DR	CD14	CD34	CD90	CD105	CD19
2	0	99.09	0.49	0	0	99.1	99.06	0
6	0	99.31	0.21	0	0.53	98.84	99.04	0
1	0	33.3	33.3	33.3	0	100	100	0

DISCUSSION

Objective 1: Development of a DMSO-free Cryopreservation Medium

The initial evaluation of a DMSO-free cryopreservation medium looked promising. In the first part of Experiment #1 both DMSO-free cryopreservation solutions appeared to sustain viable hMSCs as well or better than the control 10% DMSO cryopreservation solution. Unfortunately, because all cryopreserved conditions from that experiment looked promising, no arms or conditions were selected for further expansion directly after cryopreservation. The results from Experiment #2 suggest that at least cells cryopreserved in CPM1 would have been successful, but considering how much slower they grew than the control cells it is possible other trehalose loading strategies or cells cryopreserved in CPM2 may have performed better. Regardless, this study did demonstrate the ability to cryopreserve human cells in a fully defined medium not containing DMSO. Those cells demonstrated the ability to proliferate and express a normal profile of surface markers, CD45 -, CD73 +, HLA-DR -, CD14 -, CD34 -, CD90 +, CD105 +, and CD19 -. Further optimization of this medium should be possible. All methods of freezing used in this study targeted a 1°C/min cooling rate, but more control of the latent heat of fusion was not attempted. Detailed temperature monitoring during cryopreservation was not performed on every sample. All though the profiles that were observed both in the CRF and lyophilizer appeared normal, only blank control media solutions were used for the temperature probe, and the specific freezing profile of the experimental conditions containing cells was not directly determined. This project focused on using a very simple and repeatable cryopreservation profile largely due to the necessity of storage at -80°C post cryopreservation. A larger scale experiment using only one formulation could examine multiple freeze profiles in a CRF. An optimized freezing profile combined with subsequent storage at vapor phase liquid nitrogen temperatures would could potentially improve the results. Future work on DMSO-free

cryopreservation solutions could also focus on several other aspects. The performance of CPM2 should be evaluated in a similar manner as CPM1 was in Experiment #2, and by culturing cells post cryopreservation. Alternatively, a much larger scale experiment, or series of experiments, could be designed to optimize each component of a possible DMSO-free cryopreservation medium. This study examined using trehalose, PLASMA-LYTE A, human serum albumin, hetastarch, and polyvinylpyrrolidone as the basic cryopreservation media components. The specific concentrations of each of these components was not altered from the previously cited work, and it is possible that alternate formulations, or the addition of other potential cryoprotective agents such as proline, ectoin, or modified glucose could improve the performance of a DMSO-free cryopreservation medium.

Objective 2: Development of a Method of Long-term Storage Above Freezing Temperatures

In Experiment #1 the initial viabilities of 67.5% and 77.1% from cells lyophilized in CPM1 appeared to be very promising. Experiment #2 succeeding in reproducing this result with a viability of 74.1% from cells subjected to the same methods. Unfortunately, these cells did not seem to be capable of proliferating. In Experiment #1 the lack of proliferation could have been attributed to contamination, as every attempt to culture cells from this experiment resulted in bacterial proliferation in less than 24 hours. In Experiment #2 it seems clear that the lyophilization process resulted in damage to the cells that prevented proliferation. When cells exposed to all of the same trehalose loading conditions, formulations, and freezing, but not lyophilization, they remained capable of attachment and proliferation. No signs of contamination were observed in any flasks seeded from experiment #2 and all flasks were

incubated in the same incubator, so it is unlikely that any external factors contributed to a lack of attachment. Despite exhibiting a substantial population of viable cells, the fact that the expected surface markers were difficult to detect, or not present, may indicate that the lyophilization process somehow damaged or modified the exterior of the cells in some way without rupturing the cell membrane. If the surface proteins involved with binding were damaged or somehow irreversibly bound to trehalose the similar results could be expected. If trehalose internalized by the cell was a source of some metabolic or functional disorder then the yield of total viable cells from Arms 1 and 2 in Experiment #2 should be lower than any other arm in that experiment as they were exposed to high concentrations of trehalose for 24 hours prior to harvest. In fact, this hypothesis appears to be supported. The cells that were exposed to trehalose loading prior to harvest in Arm 1 and Arm 2 yielded between 13% and 17% fewer cells per flask harvested relative to the arms that had not been exposed to trehalose. This is a relatively small difference though, and the flasks exposed to trehalose loading were also exposed to additional manipulations. To help confirm if trehalose is having an impact on cell growth, the control flasks in future experiments should be manipulated in a similar manner and time outside of the incubator as experimental flasks. The impact of trehalose loading on cell health and future proliferation should continue to be examined, but the presence of trehalose in culture and preservation media did not prevent cells from attaching and proliferating.

Table 8. Harvest cell count data from Experiment #2. Arms 1 and 2 were exposed to 24 hours of trehalose loading. The remaining arms were cultured without exposure to trehalose until harvest. All arms were seeded and harvested at the same time.

Experiment	Condition	Trehalose Loading	# of Flasks	TVC Yield	TVC/Flask
2	Arm 1 & 2	24 Hrs	8	$8.75x10^7$	$10.9 x10^6$
2	Arm 3 & 4	N/A	8	$1.01x10^8$	$12.6, x10^6$
2	Arm 5	N/A	4	$5.23x10^7$	$13.1 x10^6$

When samples of cells lyophilized in CPM1 were rehydrated from each arm of Experiment #1, Arm C exhibited significantly higher viability than any other experimental arm. The highest viability recorded for this particular condition was 77.1% using method 2 for rehydration from cells formulated in CPM1. Using the same conditions, but preserved in CPM2, the highest recorded viability was 63.7%. After nearly two years of storage at 2-8°C, these cells preserved in CPM2 were rehydrated, and found to have maintained a viability of 55.0%. The next highest recorded viability was from Arm A preserved in CPM1 at 35.3%. Rehydration data does not exist for Arm A prior to this time point, but with the exception of Arm B cells preserved in CPM1, all conditions exhibited viability above 90% after the initial cryopreservation and prior to lyophilization. The observed drop in viability of Arm C from 63.7% to 55.0% after extended storage could suggest that even after lyophilization some degradation is still occurring. Additional samples and a larger number of replicates would be needed to more definitively evaluate the rate of viability decay over time.

It would appear that trehalose loading does have a beneficial impact on cell viability post lyophilization. In Experiment #1, cells exposed to trehalose loading by extended culture, Arm C, consistently exhibited higher viability than those exposed to other forms of loading, Arms A and B. In Experiment #2 the cells that experienced trehalose loading in Arms 1 and 2 both exhibited higher viability than the corresponding arms 3 and 4 that did not experience loading, 74.1% and 93.1% verses 66.0% and 88.8% respectively. This demonstrates that some exposure to trehalose prior to formulation can have a positive impact on cell viability. However, trehalose loading does not appear to be the most critical component to a cellular preservation strategy. When comparing data from Experiment #1 and #2, the results suggest that that cryopreservation

medium is the most critical component, and in some cases trehalose loading could be detrimental to the cell. Arm 3 in Experiment #2 was not exposed to any form of trehalose loading and exhibited a viability of 66.0% after rehydration. This is not as high as the related Arm 1 result of 74.1% viability from cells that were exposed to trehalose loading, however it is considerably higher than any viability recorded from any condition from Arm A, 25.5% viable, or Arm B, 16.4% viable, in Experiment #1. Through from different experiments and time points, all of these cells were cultured under the same conditions but Arm A was exposed to ATP as part of trehalose loading and Arm B was exposed to DMSO as part of trehalose loading, whereas Arm 3 was not exposed to loading of any kind. This could suggest that the loading methods could have been more detrimental to cell health after lyophilization than having no exposure to trehalose loading at all. Collectively this data supports the hypothesis that trehalose can be beneficial to the long-term storage of human cells, however the methods of delivery can be more damaging post-lyophilization than the benefits. Unfortunately this study was not able to directly examine trehalose internalized by any cells. It is possible that neither of the loading strategies used in Arm A or Arm B were successful in internalizing trehalose within a significant number of cells. Though viability post cryopreservation seemed good for both Arm A and Arm B it is possible the added manipulations involved with ATP mediated loading and the use of DMSO to induce pores in the cell membrane had negative effects that were only evident after lyophilization. It is unlikely the poor viability results are related to trehalose, as exposure to trehalose in the growth media appeared to have a beneficial effect on Arms 1 and 2 in experiment #2 regardless of cryopreservation or lyophilization.

It should also be noted that cells lyophilized in CPM2 were only seeded after experiment #1 and these cultures were all contaminated. A logical next step would be to repeat experiment

#2 using CPM2 to generate sterile samples, then attempt rehydration and expansion of those samples as well as surface marker analysis. In fact the data generated from the control condition may suggest that CPM2 was actually more protective than CPM1. Overall, the performance of CPM2 in terms of viability was very similar to CPM1 so continued evaluation of preservation media incorporating PVP is still reasonable.

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