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Intracellular ice formation in adult stem cells in the presence of polyvinyl pyrrolidone

Avishek Guha
Louisiana State University and Agricultural and Mechanical College, aguha1@tigers.lsu.edu

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INTRACELLULAR ICE FORMATION IN ADULT STEM CELLS IN THE PRESENCE OF POLYVINYL PYRROLIDONE

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in Mechanical Engineering in The Department of Mechanical Engineering

by
Avishek Guha
B.E., Jadavpur University, India-2003
December 2009
Dedicated to
the loving memory of my beloved father –
Subhash Guha (1939-1992)
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ABSTRACT

The main objective of this work was to assess the effect of 10% (w/v) polyvinylpyrrolidone (PVP) on the pattern of intracellular ice formation (IIF) in human adipose tissue derived adult stem cells (ASCs) in the absence of serum and other cryoprotective agents (CPAs). Passage 1 (P1) ASCs were cultured, washed and suspended in either 1x PBS (Phosphate Buffered Saline) or 10% w/v solution of PVP in 1x PBS. The freezing experiments were carried out using a fluorescence microscope equipped with a Linkam™ cooling stage using two different temperature/time cooling protocols. Both the cooling protocols had a common cooling ramp: cells were cooled from 20 °C to –8 °C at 20 °C/min and then further cooled to –13 °C at 1 °C/min (during which the extra-cellular medium froze very rapidly and was accompanied by the formation of intracellular ice in ~96% of the cells, as noted by visible “flashing/darkening”). At this point we employed either, cooling protocol 1: the cells were cooled from –13 °C to –40 °C at a pre-determined cooling rate of 1, 5, 10, 20 or 40 °C/min and then thawed back to 20 °C at 20 °C/min; or cooling protocol 2: the cells were re-warmed from –13 °C to –5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20 or 40 °C/min to –40 °C. Almost all (>96%) of the ASCs frozen in 1x PBS and protocol 1 exhibited IIF whereas almost none (<5%) of the ASCs frozen in 1x PBS and protocol 2 exhibited IIF. The lack of IIF in cells cooled in 1x PBS and protocol 2 was due to the initial loss of cell viability (confirmed through an additional membrane dye exclusion study) that was associated with the formation of IIF in the common cooling ramp, described earlier. Similarly, almost all (>95%) of the ASCs frozen in 10% PVP in PBS and protocol 1 exhibited IIF where as ~0, ~40, ~47, ~67 and ~100% of the ASCs frozen in 10% PVP in PBS and protocol 2 exhibited IIF at a cooling rate of 1, 5, 10, 20 or 40 °C/min, respectively. The observed increase in the % of ASCs exhibiting IIF when frozen in 10% PVP
and protocol 2, is presumably due to PVP mitigating the damaging effects of IIF during the common cooling ramp.
1. INTRODUCTION

1.1 Cryobiology

The word ‘cryobiology’ comes from the Greek words ‘cryos’ or cold, ‘bios’ or life and ‘logos’ or science. Hence, cryobiology is a branch of science that investigates the effects of freezing on biological systems like individual cells, tissues, organs or even whole organisms. There are various fields of study within cryobiology itself. The major few of these are a) cryopreservation – where biological materials like tissues, cells, gametes or embryos are frozen for long term storage to be used later for reproduction or continuation, b) organ preservation at hypothermic conditions, c) cryosurgery – the killing of unhealthy tissues using cryogenic fluids, d) lyophilization or freeze drying of pharmaceuticals and e) study of cold-adaptation of different organisms.

1.2 Cryopreservation: A Brief History and Theory

At a microscopic level, life depends on the movement of molecules – movements that carry out various bio-chemical reactions required to sustain processes that support a living organism or any biological system. Freezing a biological system arrests these molecular movements and hence ‘pauses’ the reactions thereby pausing ‘biological time’. If the processes of freezing and thawing back can be carried out without causing injury to the system then they can be frozen and preserved for prolonged periods of time. Cryopreservation precisely aims at doing that. The technique of storing food and other perishable commodities by freezing and drying them has been in use since historical periods. Some pioneering work in cryopreservation was done by Italian scientist Lazzaro Spallanzani in 1776 [1]. He had frozen stallion sperms in snow and noted the recovery of sperm motility on re-warming. By the late 20th century, cryopreservation had come a long way. As already reviewed by Mazur [2] and McGrath et al [3] many biological
systems and cell types have been successfully cryopreserved by that time. These include cell types like lymphocytes, red blood cells, hepatocytes, gametes, bone marrow, heart, kidney and even skin. Other than these, major works were also done by Bernard and Fuller [4] in human and non human mammalian oocyte preservation, cryopreservation of plants like algae by Walsh [5], rat and human liver slices by Day et al [6] and also spermatozoa of horses and lymphocytes of human beings by Devireddy et al [7,8].

While freezing samples of cells taken in suspension of an isotonic solution, at finite cooling rates instead of infinitesimal cooling rates, the temperature of the extracellular solution drops below the equilibrium freezing point of the solution and hence super-cools. Using a seeding agent like a chilled needle or sometimes some commercially available bacteria like pseudomonas syringae can help ice nucleation in the extracellular medium at this point. Even without a seeding agent ice typically forms in the extracellular medium first albeit at a lower temperature [56]. The precipitation of pure ice from the extracellular medium increases the concentration of the unfrozen fraction of the medium and sets up a chemical potential difference between the supercooled unfrozen cellular cytosol (which remains at a higher chemical potential) and the extracellular medium. This leads to beginning of water efflux from the cell through the cell membrane into the extracellular space. From here onwards one of two things may happen - i) if the cooling rate is too fast, the cells may not have enough time to undergo dehydration and due to the continuously dropping temperature and the availability of freezable water inside the cell, the cell may freeze internally (also known as intracellular ice formation or IIF) or ii) if the cooling rate is slow enough, the cells get enough time to lose enough water (also known as water transport) thereby maintaining a chemical potential equilibrium with the solution outside the membrane and avoid IIF. Both these phenomena – IIF and water transport, have important
implications on post-thaw cell viability. IIF is commonly known to be one of the chief causes of cell death and has also been shown to be intimately and negatively correlated to cell survival [9-12], whereas an excessive dehydration of cells too has been shown to be lethal due to ‘toxicity effects’ of intracellular solutes and electrolytes [13]. However, the best survival rate has been found in general when the cell retains ~ 5% of its water volume and avoids freezing [49]. The rate at which water effluxes from the cell is not only dependant on the concentration gradients of solutes across the cell membrane but also on the permeability of the cell membrane to water at any point of time. The membrane is assumed to be a semi-permeable that prevents the migration of solutes across it but lets water to flow across it. The value of permeability ($L_p$) varies from cell to cell and it is hence important to measure the value of $L_p$ to understand and construct a freezing protocol that is neither too slow to induce ‘toxicity effects’ nor too fast to create IIF. The optimal cooling rate required for any type of cell to produce the maximum post-thaw viability is hence dependant on the composition of the extracellular medium as well as the type of cell being used. The general shape of cooling rate (x-axis) against cell viability (y-axis) is a inverted U-shaped curve [14] with the exact geometry of the curve being dictated by the factors stated above.

In 1949 it was discovered that addition of glycerol to the extracellular space provide significant protection to sperm cells during cryopreservation [15]. Since then most cryopreservation protocols use the protective properties of such additives during freezing by introducing them in the freezing medium as a cryoprotective agent (CPA). Broadly speaking, CPAs can be divided into 2 major categories – permeating and non-permeating. Permeating CPAs such as ethylene glycol (EG), dimethyl sulfoxide (DMSO), propylene glycol (PG) and glycerol diffuse into the cells when added to the freezing medium. Non permeating CPAs are mostly polymers like polyvinyl pyrrolidone (PVP), hydroxyl ethyl starch or sometimes sugars
which cannot diffuse into the cells. The exact mechanism as to how the cells are protected by the additives are yet to be ascertained. However, it is postulated that [16] permeating CPAs reduce the concentration of the harmful electrolytes during slow freezing dehydration and thereby protects the cell from its effects. It has also been thought to preferentially exclude cell proteins from their hydration shell and entropically stabilizing them [17,18]. IIF is reduced due to the colligative properties of the CPAs. When used in high concentrations, they can increase the viscosity of the solution thereby impeding the growth of ice crystals into the cells and reduce chances of ice nucleation. However, exposure to cryoprotectants for a long period of time can be harmful for cells because of their toxicity, particularly if CPAs are used in high concentrations. Also cryoprotectants need to be removed before the cells are used [16]. But the addition and removal of cryoprotectants can exert significant stress on the cells. When a cell preserved in cryoprotectants is thawed and placed in an isotonic solution, the cell initially swells as water enters the cell at a rate faster than the CPA can flow out of it. This may lead to the cell membrane being stretched beyond damage. Hence the CPAs are removed in a stepwise manner so that the cells are not damaged. However, this stepwise removal of CPAs means a longer time of exposure to the toxicity of cryoprotectants. Hence these 2 factors (long exposure time to CPAs and washing off the CPAs in isotonic solution) need to be balanced to ensure survival of cells.

1.3 Cryomicroscopy and Differential Scanning Calorimetry

The methods of studying the behavior of biological systems during cryopreservation are “differential scanning calorimetry” and “cryomicroscopy”. In differential scanning calorimetry the amount of heat required to be supplied or withdrawn to or from a sample, so that the sample temperature approaches a “reference temperature”, is measured as a function of temperature. The DSC technique is primarily used to measure quantities like enthalpies of phase change, reaction
kinetics, heat capacities, glass transition temperatures, thermal history, decomposition effects, and purity of solid samples. The two main types of DSC are the power compensated DSC and heat flux DSC. Both measure heat applied, but the power compensated type holds the temperature to a preset value while the heat flux type holds the heat applied constant. The DSC technique has been used elsewhere previously to measure cellular dehydration or water transport [8, 19-21].

In cryomicroscopy, samples are loaded on a ‘cryostage’ mounted under a light microscope. The ‘cryostage’ is fitted with a liquid nitrogen tank and pump and the setup is used to cool the samples. The changes occurring during the freezing process can be observed under the microscope and images of the changes can be recorded. The availability of computerized image enhancement technique helps in overcoming the low resolution of video recordings. This has been used to study freezing responses of various cells like those of plants and mammals. One major assumption in cryomicroscopy is that visible area of the cells, though two dimensional in nature can be thought of as a projection of a three dimensional volume of the cells. Hence volumetric changes of only spherical cells can be studied under cryomicroscopes. If cells are not spherical, the visible / projected dimension of the cell cannot be used to calculate the volume of the cells during the analysis. Under this assumption the technique is very useful to study cellular dehydration by visible volume shrinkage. It is also useful for studying IIF occurring in cells. During IIF, the light cannot pass through the cells as the many surfaces of the crystals of ice formed inside the cells reflect light away and the cells becomes effectively opaque and darkened. Hence, the occurrence of IIF is evident by sudden darkening of cells which is also sometimes called ‘flashing’.
1.4 Overview of the Thesis

This work evaluates freezing response of adipose tissue derived passage 1 (P1) adult stem cells (ASCs) when suspended in either of 10% (w/v) polyvinyl pyrrolidone (PVP) with 1x phosphate buffered saline (PBS) or only 1x PBS. Adult stem cells are undifferentiated cells that can be found throughout the body. These cells multiply by cell division and have the capability of replacing dying cells and rejuvenating damaged tissues. They can be found in juvenile as well as adult humans and animals. A lot of scientific interest has been generated around these cells as they have the capacity to produce all the cells of the organ from which they arise. Adipose tissue derived adult stem cells are stem cells which come from fat. Liposuction fat wastes from hospitals are digested in stem cell labs. After processing the tissue, initially stromal vascular fraction (SVF) cells are obtained which contain not only stem cells but also other kind of cells like blood cells, fibroblasts and endothelial cells. However, successive passaging for several generations yields homogenous adipose stem cells [25, 44, 58-61].

The cryopreservation protocols of ASCs in general contain the use of fetal bovine serum (FBS) and DMSO (a very commonly used cryoprotective agent). In simple terms, FBS is the plasma that remains after coagulation of blood drawn from an unborn bovine fetus. However, the use of FBS is plagued with problems like batch-to-batch inconsistency [22-25] in serum quality, its susceptibility to bacterial contamination and sensitivity to degradation and adsorption. FBS is also relatively costly. Apart from these practical difficulties there are moral and ethical concerns regarding the collection of serum from living animal. On the other hand, DMSO, though a commonly used CPA, is also not free from controversy regarding in vivo applications in humans. As of now the FDA approves the use of DMSO only in the treatment of interstitial cystitis. Hence in this work was primarily aimed to devise a cryoprotective protocol that avoids the use of
FBS as well as DMSO during the cryopreservation of ASCs. A recent study by Thirumala et al [26] has shown that 10% PVP offered some level of cryoprotection during the freezing of ASCs. However, the post-thaw survival was only measured to be around ~65%. The present work investigates the occurrence of IIF in ASCs with and without the use PVP and sheds light on the effectiveness of PVP as a CPA for ASCs as well as provides an optimized protocol that produces the minimum degree of IIF during cryopreservation.
2. LITERATURE SURVEY

2.1 Polyvinylpyrrolidone (PVP) as a Cryoprotective Agent

Since the discovery of glycerol’s cryoprotective properties in 1949, most cryopreservation protocols have used different additives that lend similar cryoprotective properties to the extracellular medium during freezing of cells. The use of polyvinyl pyrrolidone as a cryoprotectant was being studied as early as the 1960s. Most of these were in relation to freezing of blood. Meryman pointed out in 1968 [27] that when rapid freezing of blood with glucose as a CPA was reported [28] it was then hoped that the use of the CPA in small concentration would mean that the thawed blood can be directly infused thereby eliminating the step that requires washing off the CPA as this particular step had been plagued with problems when it was already being used with glycerol. The washing of the cells from the CPA induces a lot of osmotic stress on cells which can damage them. However, glucose was found to enter the cells thereby rendering the cells hypertonic leading to intravascular hemolysis on infusion. Once glucose failed to serve the purpose, pure lactose was tried out as a CPA. However, the concentrations required to avoid freezing hemolysis was found to be too much which caused osmotic damages in the cells.

In 1961, Doebbler et al [29] published a study where they reported the effective use of PVP in the cryopreservation of rabbit blood. In the study, rabbit blood was collected acid-citrate glucose and PVP of molecular weight 40000 mixed with saline was used to make a 7% solution. The mixture were taken in capped aluminum tubes and frozen rapidly by shaking in liquid nitrogen. They were stored at -170°C for several days before thawing rapidly in water bath at 45°C. It was shown that the process achieved recoveries of greater than 90%. Even when the red blood cells were infused directly, with the polymer, 93% of the cells exhibited 24-hr survival.
In another study in 1963, Persidsky et al [30] compared the effectiveness of DMSO and PVP in the survival of bone marrow cells. Bone marrow cells were obtained from femurs of rats of an inbred Long-Evans strain 6-8 month olds. The cells were frozen using different concentrations of DMSO or PVP in Hank’s solution. The samples were either frozen directly or kept for various length of time before freezing. The cooling rate used was 1°C/min with ice being seeded at -5°C. As soon as -25°C was reached, the samples were transferred to an alcohol bath at -79°C and kept for 30 minutes. During thawing, they were rapidly thawed by immersing them into a water bath at 37°C. After that the samples were washed in Hank’s solution and centrifuged at 900 r.p.m for 5 minutes. The cells were then resuspended in Hank’s culturing medium containing 10% PVP and 33% rat serum. Assessment of cell viability was carried out using phase microscopy. The study showed that as a preservative, DMSO provided maximum survival of 50% at 10% concentration. The viability declined sharply if the concentrations were a little bit higher or lower (12.5% or 7.5%). Also, the cells that were equilibrated for 10 min as opposed to 30 or 60 minutes, showed maximum survival. Longer exposure times yielded lower viabilities. Also, the increasing toxicity of DMSO with concentration was ascertained by the increasing viability rates with decreasing concentrations of DMSO used (3, 1,0.5 and 0.1%). When PVP was compared, it was found that PVP has no significant change in viability percentages even when the concentration was increased from 10 to 20%. The broader optimal range was attributed to the non-toxicity of PVP. In addition, equilibration period was not required with PVP as it does not penetrate the cells. Since PVP was non toxic, there was no need of removing it from the samples after thawing. The highest survival rates found with PVP was around 30% which was lower than the numbers found with DMSO. The authors concluded that although DMSO provided better survival percentages, the variability was too much and required
a lot of precaution while handling the CPA. In that context they preferred PVP to DMSO as it produced much more predictable results and was simpler to handle.

In 1975, Zdebska et al [31] made a comparative study of glycerol and PVP by assessing the phagocytic ability of granulocytes, ability of blastic transformation of lymphocytes and the viability of the number of these cells before freezing and after thawing. There were no significant differences found in the quality of granulocytes stored in 13% glycerol and 15% PVP. However, the results obtained were much worse off when compared to similar studies with DMSO.

In a work by Richards et al published in 1961 [32], it was showed that PVP of 30000 molecular weight provided about 30% protection against injuries during freezing and thawing which was better than that provided by glycerol. In 1962 [33], the team of Richards and Persidsky used bone marrow cells to study the mechanism by which PVP offers protection during freezing and thawing. It was clear by then that diffusion was not a method by which PVP may enter the cells just because of the large size of its molecules. To test whether pinocytosis was a method in which PVP was being carried, if at all, into the cells, they used iodine-131 labelled PVP with phosphate salts to carry out experiments on rabbit bone marrow cells. They established that the presence of phosphate ions did actually help in pinocytosis. In the second part of their work they also established that the maximum survival of cells against freezing injury was when they were frozen without phosphate salts or when pinocytosis did not occur. So, evidently, the protection offered by PVP during the freeze-thaw process was overwhelmingly extracellular. The possible ways in which PVP acted as protective additive was thought to be by adsorption and dilution of electrolytes, by occlusion of pores that prevent intracellular ice-seeding and enhances supercooling, reduce excessive intracellular dehydration and by restoration of cell permeability after thawing.
In a study [34] in 1970, Mazur et al showed that using 15% PVP offered about 35% survival values in mouse bone marrow cells, when frozen to -196°C at a velocity of 10°C/min. In tissue culture, Chinese hamster cells when frozen with 15% PVP produced 60% of the survival values of controlled unfrozen cells.

In a very recent study in 2009, Kim et al [35] equilibrated mouse 2-celled embryos were with 4% (v/v) EG at 37°C for 15 min and then exposed them to vitrification solutions containing varying concentrations of PVP (5.6 & 7.5%) and 0.4M sucrose for 5 min at 37°C. For recovery, embryos were transferred one after another into 300 µl of 0.5 M & 0.3 M sucrose at room temperature for 5 min and then into M2 medium at 37°C for 10 min. Embryos were washed three times and then cultured in KSOM medium under mineral oil at 37 °C for 96-120 hr in 5% CO2 and 95% air at maximal humidity. It was found that the survival rate of the 7.5% PVP concentration group was significantly higher than the 5% and 6% PVP groups. Also, the survival rates of the 7.5% group was similar to that of the control group.

In the present study we have used the advantage of the non-toxic and the cryoprotective property of PVP in conjunction with an unorthodox freezing protocol to create a DMSO-free, serum-free cryopreservation protocol for adipose tissue derived adult stem cells.

2.2 Previous Work with Adult Stem Cell Cryopreservation

Most multi-cellular organisms contain stem cells. They are some of the main building blocks of the organism. Stem cells can renew themselves by mitotic cell division and differentiate into different cell types. Hence it provides a wonderful avenue to study cellular and developmental processes. Understanding the processes involved in differentiation of stem cells into multi-lineage pathways can help develop novel strategies for organ regeneration and transplantation [36-40]. Thirumala et al [41] carried out a study to find out the effect of different
freezing parameters on the immediate post-thaw membrane integrity of adipose derived adult stem cells. The parameters studied were cooling rate, end temperature, hold time and thawing rate for 2 different levels for each parameter – high and low. The high and low values for each parameter were as follows: cooling rate - 1 and 40°C/min, end temperature - -80 and -20°C, hold time – 1 and 15 minutes, and thawing rate 10 and 200°C/min. The authors concluded that for 99% confidence level only cooling rate and end temperature had a significant effect on cell membrane integrity for all passages of the cells. The increase in cooling rate had an adverse effect while increase in end temperature had a beneficial effect on post thaw cell membrane integrity for passage 0 (P0) cells. Although small as compared to cooling rate and end temperature, hold temperature and thawing rate has a significant effect on P3 cells.

In a study by Fuller et al [42], passage 0 (P0), passage 1 (P1), and passage 2 (P2) were cooled at 1, 5, 20 or 40°C/min to -80°C either with or without a CPA (DMSO) using a directional solidification stage (DSS). The cells were then thawed back and exclusion tests were carried out with fluorescent dyes. It was found that cells frozen without DMSO had a lower post-thaw viability than cells frozen with 10% (v/v) DMSO. Not only that, it was also found that cells frozen in a commercially available control rate freezer had a better post thaw viability as compared to cells frozen in a DSS. The reason for that was thought to be differences in nature and damaging effects of ice-crystals formed in a DSS as compared to a control rate freezer.

With an aim to improve cryopreservation protocols so as to reproducibly maintain ASC viability and multipotentiality, Goh et al [43] studied the efficiency of conventional DMSO cryopreservation protocol by measuring differentiation potential after one freeze cycle. It was found that cryopreservation had ‘little to no effect’ on the efficiency of the cells to adhere to the flasks and to form a fibroblast population, or to differentiate into mature adipocytes after
induction. However, they also found that post thaw viability was a function of storage concentration and the optimal concentration was $0.5 \times 10^6$ cells per ml of cryopreservation medium.

In order to ascertain the values of reference cell membrane permeability and activation energy, Thirumala et al [44] used the DSC technique to study water transport in SVF (stromal vascular fraction) cells and ADAS (adipose tissue derived adult stem) cells for passages 0 and 2. Volumetric shrinkage of the ASCs were carried out in the presence of extracellular ice at 20°C/min with either of 10% DMSO or 10% glycerol. Modeling ASCs as spheres of diameter 50 µm and with and osmotically inactive cell volume of 60% of the isotonic cell volume a model of water transport was fitted to the experimentally obtained data. In the presence and absence of CPA, the values of $L_{pg}$ (membrane permeability) ranged from $23.1 - 111.5 \times 10^{-15}$ m$^3$/Ns and the values of $E_{lp}$ (activation energy) ranged from 43.1 – 168.7 kJ/mol.
3. MATERIALS AND METHODS

3.1 Measuring Cellular Response to Freezing Stress

In 1963 Peter Mazur successfully came up with a model [9] that could describe and quantify the cellular response to general freezing stress i.e. the change of volume of cells due to loss of cytoplasmic water to the extracellular medium containing ice. However, in his model, Mazur assumed that the cell membrane’s permeability is temperature-independent. This assumption was later modified by Levin et al in 1976 [45] where the permeability of the membrane was assumed to be temperature dependant. The major assumptions of Mazur’s model are as follows:

1) The extracellular space is considered infinite.

2) The cells are assumed to be spherical. So, a volume of a cell is \( V = \frac{4\pi r^3}{3} \).

3) The surface area of the cell is considered constant and equal to the original cell membrane area = \( 4\pi r_0^2 \). Where \( r_0 \) is the initial cell radius. [47,49].

4) Intracellular medium is acts as a dilute ideal solution and follows Raoult’s law of solutions.

5) The latent heat of vaporization of water is constant in the temperature range of interest.

6) The hydrostatic pressure across the cell membrane is zero (this holds good for mammalian cells).

Summarily the water transport equation is:

\[
\frac{dV}{dT} = -\frac{L_p A_c R T}{B v_w} \left( \ln \frac{\mu_i^w}{\mu_o^w} \right) \ldots \ldots \ldots (1)
\]

Here \( V \) is the cell volume, \( T \) is the absolute temperature, \( L_p \) is the permeability of cell membrane to water, \( R \) is the gas constant, \( B \) is the cooling rate, \( A_c \) cell membrane surface area that takes
part in water transport, \( v_w \) is the partial molar volume of cell water, \( \mu_o^w \) and \( \mu_i^w \) are the chemical potential of extracellular liquid and intracellular liquid respectively. The extracellular solution is assumed to be composed of water and solutes like sodium chloride. Also, the solution is in equilibrium with the extracellular ice that forms. Hence this mixture of ice and salt-water solution is modeled using the equilibrium properties for a solid-liquid solution. Using the Gibss-Helmholtz equation that relates the activity of a solution to the function of temperature, we can write:

\[
\frac{\partial (\ln \mu_w)}{\partial T} = \frac{\Delta L_f}{RT^2} \ldots \ldots \ldots \ldots (2)
\]

If we integrate equation 2 within the temperature limits of 0°C (or 273.15 K) and any temperature \( T \) in our range of interest, we get,

\[
\ln \mu_w = \frac{\Delta L_f}{R} \left[ \frac{1}{T_R} - \frac{1}{T} \right] \ldots \ldots \ldots \ldots \ldots (3)
\]

where \( T_R \) is the reference or the phase change temperature for pure water (273.15 K). Since by assumption 4 the intracellular solution is modeled as an ideal solution the chemical activity of the solution can be replaced by the mole fraction of water, or,

\[
\mu_i^w = x_i^w \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots

In equation 5, the value of \( \phi_2 \) is 2 which is the dissociation constant for sodium chloride, \( n_s \) is the number of moles of solutes in the cell. In the numerator, \((V-V_b)\) represents the total osmotically active cell volume at any point of time, \( V_b \) being the osmotically inactive cell volume. So, using
equation 3 and 5 and substituting them in equation 1 Mazur’s equation of water transport can be derived as:

$$\frac{dV}{dT} = -\frac{L_p A_c RT}{B V_w} \left[ \ln \left( \frac{V - V_b}{(V - V_b) + \nu_w (n_s \phi_s)} \right) - \left( \frac{\Delta L_f}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right) \right) \right] \ldots \ldots \ldots (6)$$

In 1976, however, Levin et al came up with a temperature dependant expression for membrane permeability ($L_p$). It was expressed as an Arrhenius relationship as follows:

$$L_p = L_{pg} \exp \left[ -\frac{E_{lp}}{R} \left( \frac{1}{T} - \frac{1}{T_R} \right) \right] \ldots \ldots \ldots (7)$$

In this expression, the $L_{pg}$ is the permeability of the membrane to water at a reference temperature ($T_r = 273.15$ K) and $E_{lp}$ is the activation energy for the permeability process to start.

3.2 The Optimal Cooling Rate

As mentioned in the previous sections, cooling rates categorized as “too high” or “too low” depends on the cell type. The damages to cells in such cases are either by IIF or by “solution effects” respectively. So, the cooling rate required for the maximum cell survival is somewhere between the “high” and “low” rates. For various cell types, these rates have been experimentally found out and plotted and the curves take an inverted U shape. Figure 3.1 shows the representative survival curve for any cell type in general. It can be seen that increasing the cooling velocity increases the survival percentage till it reaches a maximum and after that the increase in cooling velocity results in a drop of survival percentage. It had already been found that the value of the optimal cooling rate for a cell type depends on various parameters like $L_{pg}$, (reference membrane permeability), $E_{lp}$ (activation energy), $V_b$ (inactive cell volume), $V_o$ (initial cell volume) and $A_c$ (surface area of the cell). Since these values change from cell to cell, the optimal cooling rates too vary immensely from cell to cell – e.g. the optimal cooling rate for cells
like bone marrow is ~ 1°C/min where as that for red blood cells is ~ 1000°C/min [13,48]. In

![Diagram showing the relationship between cooling velocity and cell viability.](image)

**Figure 3.1 Optimal Cooling Velocity Curve: A inverse “U”-shaped curve showing that effects of too "slow" cooling as well as too fast cooling being deleterious for cells.**

2005, Thirumala and Devireddy [49] came up with a much simpler way to ascertain the optimal cooling rate for a given type of cell when cell level parameters such as $L_{pg}$, $E_{lp}$ and $A_c/WV$ is known a priori. Here the parameter $A_c/WV$ combines two other cell level parameters into one single parameter – this parameter is nothing but the ratio of the initial cell surface area to the initial osmotic water volume ($WV = V_o - V_b$) inside the cell. In the work, ‘optimal cooling rate’ was defined as the cooling rate that trapped 5% of the initial water volume inside the cell at a temperature of -15°C. Then equations 6 and 7 (water transport equations) were numerically solved using a fourth order Runge-Kutta method to calculate the values of optimum cooling rate over a range of different key cell level parameters namely $L_{pg}$, $E_{lp}$, $V_b$, $T_{end}$ (end temperature), D (diameter of cell) and the ratio of available surface area to initial volume of intracellular water
(A_c/WV). Following the investigations of the variation of optimal cooling rate (B_{opt}) with these parameters it was found that the B_{opt} varies exactly linearly with L_{pg} and A_c/WV values. A graph representing the variation of B_{opt} (along Y-axis) with A_c/WV (along X-axis) was plotted with L_{pg} value kept constant at 1µm/min-atm. A family of such curves were obtained for different E_{lp} values. These curves were then collapsed into a single plot of B_{opt} vs. E_{lp} with the A_c/WV ratio kept constant at 1.0 which produced the Generic Optimal Cooling Rate Chart (GOCRC). GOCRC predicts the ‘optimal cooling rate’ of a biological system which will have L_{pg} = 1µm/min-atm and A_c/WV =1.0, provided E_{lp} is known beforehand. Since the variation of the ‘optimal cooling rate’ with the cell membrane permeability (L_{pg}) and surface area to volume of initial intracellular water (A_c/WV) was already known to be linear, a new equation that produces the ‘optimal cooling rate’ (B_{opt}) for any such biological system with L_{pg}, E_{lp} and A_c/WV known a priori, could be written down as:

\[ B_{opt} = B_{GOCRC} \, (L_{pg})_a (A_c/WV)_a ......(8) \]

where B_{GOCRC} is the cooling rate as read from the GOCRC chart for the measured E_{lp} and (L_{pg})_a and (A_c/WV)_a are the measured values of reference cell membrane permeability and ratio of surface area of cell to initial osmotic intracellular water volume. The ‘optimal cooling rate’ values obtained from the equation was compared with experimentally determined values published in literature. There was a reasonably good agreement between the two with the exception of only AT-1 cells (a tumor cell line).

3.3 Cryomicroscopy

The experimental technique used here was cryomicroscopy. As touched upon in the previous sections, this method employs the use of light microscopy to visualize cells loaded on to a sample holder called the “cryostage” which can be cooled or heated by the use of a liquid
nitrogen pump or heater used in conjunction respectively. In his book “Through the microscope: science probes an unseen world” in 1965 [50] author M. D Anderson, writes about the history of microscopy. In the 1600’s, Anton van Leeuwenhoek of Holland used tiny lenses of great curvature to make significant discoveries in the field of biology. With his lenses Leeuwenhoek saw and described bacteria, yeast plant and even the abundance of tiny life that can be seen in a mere drop of water. Later Robert Hooke of England developed on the Leeuwenhoek’s crude lenses and confirmed many of his findings.

In the 1800s, premier botanist Julius Sachs, who was interested to learn about the fate of plant tissues when subjected to freezing stresses, came up with probably the first notable application of cryomicroscopy [51]. Sachs’ Phd. student Herman Muller-Thurgau also worked on similar lines researching cellular response to freezing stress and super-cooling.

However, one of the most significant advancement in the field of cryomicroscopy came from Dr. Hans Molish who built his very own cryomicroscope from a wooden box. In 1897, he both the cavity and the walls were closed by a lid which had openings to allow adjustments of the microscope. The lid was in contact with a thermometer which recorded the temperature. Once the microscope and the freezing mixture was placed inside, and the lid closed, other visual aid operations like focusing or adjusting were done with the fine adjustment provisions provided, as described above. It has to be noted here that this apparatus did not have thermal control. But the significant improvement at this point was the fact that it saved the observer from inflicting himself/herself to severely low temperature for such work. Before this, most of the work done was where the sample, stage assembly, microscope and the observer were in cold environment. Cohn (1871), Kunisch (1880), Weigand (1906) and even Julius Sachs (1892) used naturally cold
environments (being outdoors or holding their microscope in the window) to study freezing processes [53].

The works of Diller and Cravalho [54] in the late 1960’s heralded the modern generation of cryomicroscopy. They used a closed loop feedback control system capable of preprogramming and independent regulation of the specimen cooling rate and instantaneous temperature. It was designed to have a small thermal mass in conjunction with an analog electronic control circuit to facilitate a rapid response time constant. Cold and dry nitrogen gas which passed below a thin glass plate on which the specimen was mounted, was used to cool the unit. Heating was achieved by applying electric voltage across a thick film coated applied to the bottom side of the plate. Although the cooling load was approximately steady state the heating could be modulated quickly by varying the electrical voltage from the controller. The temperature of the sample was monitored by a thermocouple inserted inside the cell suspension. A simple proportional control logic was applied via the hardwired circuitry of the analog system to the thermocouple input signal in comparison with a preprogrammed electrical representation of the desired specimen thermal history. Simultaneous heating and cooling could achieve thermal transients to 7000 °C/min. This enabled a comprehensive study of the ice nucleation in human erythrocytes [54, 55].

3.4 The Experimental Setup

The experimental setup (Fig 3.2) that was used was very similar to that described elsewhere [56]. Briefly, the functions of sample temperature control, event correlation, image storage and analysis were integrated. The volumetric shrinkage of cells was detected by noting the reducing diameter of cells due to flow of cellular water into extra-cellular space under
growing osmotic stresses, while IIF was evidenced by sudden “blackening” or “flashing” of the cells.

Figure 3.2 The Cryomicroscope: Shows the experimental setup of cryomicroscope. The different parts are labeled – A: The temperature controller, B: the liquid nitrogen pump, C: the liquid nitrogen tank, D: the cryostage, E: the microscope, F: camera.
3.4.1. Temperature Control - The Cryostage

Fig. 3.3 The Cryostage: Shows the close-up view of the cryostage. A : the window for viewing through the microscope, B : x and y manipulators for centering and adjusting the sample in the field of view.

This (Fig 3.3) is the part of the cryomicroscope that houses the sample and the sample carrier. This “cryostage” manufactured by Linkam Scientific™ (Surrey, UK, Model: BCS 196) is capable of controlled cooling and heating between –125 °C and +160 °C at cooling/heating rates ranging from 0.1 to 130 °C/min. The cryostage contains a silver block over which the sample can be placed after being confined in a high thermal conductivity quartz crucible. The temperature is measured by a platinum resistance thermocouple placed inside the silver block which also housed an electrical heater. The temperature controller powers the heater inside the stage which
is cooled by a stream of liquid nitrogen vapor that is pumped through the “liquid nitrogen pump” attached to the cryostage. The action of the heater and the liquid nitrogen pump (which is also connected to the temperature controller) forms a closed loop feedback control system that keeps the sample at the desired temperature or setpoint.

3.4.2. Temperature Control Software

We used Linksys 32 as the temperature control and data acquiring software system. The temperature controller, which is linked to a desktop computer with Linksys 32 installed in it, can be used to preprogram the thermal history of the sample. The preprogramming can also be done via the software installed in the connected personal computer. Once the desired cooling protocol is fed to the temperature controller via the software or through the controller itself, it imposes the preprogrammed thermal history (or the cooling protocol) on the sample held in the crucible by using the liquid nitrogen pump and heater as required. The software is capable of recording protocols with up to 100 different ramps. Any cooling protocol or “temperature profile” typically consists of several ramps constructed based on the discretion of the user. Each ramp can be described in terms of different cooling/heating rates, limits and hold times which can be individually fed to the controller by the software. The controller executes each ramp between the specified limits as per the cooling/heating rates and at the end of each hold time, it moves on to the next ramp till the whole protocol is completed. The Linksys software provides the flexibility to change the values of a temperature profile even while running the profile. It also provides the option to save a profile for quick setup of a similar experiment running similar protocol.

3.4.3. Image Control and Event Correlation

During cryomicroscopy, the changes that occur in a cell with respect to changing temperature or applied freezing stress is viewed through a microscope. The microscopes are
generally fitted with a camera which can continually capture images from the start till the end of the preprogrammed temperature profile. Most of the cameras have the ability to update the image very rapidly (25-30 frames/second) and record them simultaneously and can be used to make a movie stacking the images one after another in proper sequence. The image capturing softwares also provide the flexibility to slow down or speed up the movies if required. For subsequent analysis, these recorded images needs to be annotated with the corresponding time and temperature from the beginning of the cooling protocol. The cryostage in this case was attached to a microscope (Nikon Eclipse E600, Nikon Instruments, NY) which is also fitted with a Photometrics Coolsnap cf camera (Hamamatsu, Photonics, Bridgewater, NJ). During the experiments a live video signal is sent from the camera to the attached DELL personal computer and the images recorded with the help of commercially available Metacam software (Universal Imaging Corp., Buckinghamshire, UK).

3.4.4. Recording Optical Information and Using the Condenser Lens

Viewing the two important bio-physical phenomenon namely ‘water transport’ evidenced by shrinkage of cells and ‘IIF’ evidenced by sudden “flashing” depends on the proper passing of light from the source through the cells suspension contained in a transparent crucible. Hence, setting up the microscope properly for viewing and recording optical information is extremely important. Once set up, the lens system of the microscope can be used, as it is, any number of times unless somehow (like when the bulb is to be replaced) the arrangement is disturbed. A simple schematic diagram, reproduced from the Linkam Scientific Instruments Ltd. Manual (Fig. 3.4) shows the schematic of the lenses and other optical equipment inside the microscope. The details of how to work with the equipment is present in the ‘work instruction’ or manual provided by the manufacturer [57]. Briefly, these are the following steps that need to be followed
Figure 3.4 The Lens System: Shows the schematic diagram of the lens system of the cryomicroscope. (Linkam Scientific Instruments Ltd.)

to get the microscope ready for use:

1. The field diaphragm at the base of the microscope has to be opened to the largest aperture and a flat, thin piece of paper has to be placed over it.

2. The light source has to be turned on to the maximum output.
3. The lamp housing has to be slid backwards and forwards until an image of the filament appears on the paper just placed above.

4. With the help of vertical centering ring and lateral centering screw the lamp has to be adjusted to the center the filament.

5. The lamp housing has to be pulled slightly forward until the filament image is diffused, or alternatively a diffusing filter can be fitted.

As mentioned before, since the microscope was fitted with a camera, the images seen under the microscope was being continually recorded by the camera and stored in the computer attached to the system. These images were eventually used to analyze the data.

3.4.5. Image Analysis for IIF and Water Transport

Basically, 3 different methods of image analysis exist [56] namely a) the mechanical planimetry of still images, b) electronic area analysis and c) fully automatic computerized area analysis. In the first case cross-sectional area of different cells are measured manually by measuring the diameter of the cells from the images against a given scale. Paper tracing of video images is also considered to be mechanical planimetry. In the second method, a computer generated circle, the radius of which can be varied by a joystick is used by the user to match the cell diameters and thus the required radius can be read off and areas calculated. In the third method, images of cells can be converted to digital form and analyzed by a computer programmed to recognize cell boundaries and calculate the area as well. In this work we used the first method to calculate the cell diameters.

To study water transport i.e. cellular dehydration during freezing, diameters of cells at different points of time during the cooling protocol was measured. Assuming that the cells are spherical in shape, their volume was calculated to be \( V = \frac{\pi}{6} D^3 \). Using this formula the
volume of the cells at different temperatures was evaluated and then the normalized volume (ratio of volume of a cell at any particular sub-zero temperature to its original volume) was plotted against the corresponding temperature.

Similarly for IIF, images recorded by the camera were analyzed for the number of cells undergoing “flashing” or “blackening”. These numbers, as a ratio of the total number of cells present in the specimen, denoted the probability of intracellular ice formation (PIIF) which was then plotted against the corresponding sub-zero temperature for different cooling velocities.
4. FREEZING RESPONSE OF ADIPOSE TISSUE DERIVED ADULT STEM CELLS

4.1 Background

Adult stem cells can differentiate into several other cell types of the body and studying them could provide valuable insights about early embryonic development, organ regeneration and transplantation [36-40]. Fortunately, adipose tissue is an easy and abundant source for such adult stem cells and long-term storage (cryopreservation) procedures for these cells are currently being actively explored [26,44,58-61]. The use of high molecular weight polymers such as Polyvinylpyrrolidone (PVP) as the CPA of choice for ASCs has also been recently investigated [26,60,61]. PVP is known to be non-toxic and has earlier been used as a CPA during freezing of erythrocytes [62,27], mouse bone marrow cells [30,32], mouse lymphocytes [63] and Chinese hamster cells [34]. Intriguingly, Thirumala et al. [26] found that 10% (w/v) PVP provides the highest survival rates (~ 65%) for ASCs frozen over-night in an ethanol-jacketed container in a – 80°C freezer. The aim of this study was to further assess the effect of PVP on the formation of intracellular ice in ASCs and its ability to possibly mitigate the associated freezing injury.

4.2 Sample Preparation and Experiments with ASCs

4.2.1. Isolation, Collection and Culture of Cells

All human protocols were reviewed and approved by the Pennington Biomedical Research Centre Institutional Review Board. Unless otherwise stated, all reagents were obtained from Sigma Chemicals (St. Louis, MO). The method of culturing and harvesting adult stem cells has been described elsewhere [26,44,58-61]. Briefly, subcutaneous adipose tissue liposuction aspirates from three patients were provided by plastic surgeons in Baton Rouge, LA. These, tissue samples (100 to 200 ml) were washed 3-4 times in phosphate buffered saline (PBS) pre-warmed to 37 °C, suspended in PBS supplemented with 1% bovine serum albumin and 0.1%
collagenase (Type I, Worthington Biochemicals, Lakewood, NJ), and digested with gentle rocking for 45-60 min at 37°C. The digests were centrifuged for 5 min at 1200 rpm (300xg) at room temperature, re-suspended, and the centrifugation step repeated. The supernatant was aspirated and the pellet re-suspended in stromal medium (DMEM high glucose, 10% fetal bovine serum, 100 units penicillin/ml, 100µg streptomycin/ml, and 25µg amphotericin/ml). The cell suspension was plated at a density equivalent to 0.125 ml of liposuction tissue per sq cm of surface area, using a 35 ml volume of stromal medium per T225 flask. Cells were cultured for 48 hrs in a 5% CO₂, humidified, 37°C incubator. The adherent cells were rinsed once with pre-warmed PBS and the cells fed with fresh Stromal Medium. The cells were fed with fresh stromal medium every 2 days until they reached approximately 75-80% confluence. The medium was then aspirated; the cells were rinsed with pre-warmed PBS, and harvested by digestion with 0.05% trypsin solution (5-8 ml per T225 flask) for 3 to 5 min at 37°C. The cells were suspended in Stromal Medium, centrifuged for 5 min at 1200 rpm (300xg), the pellet re-suspended in a volume of 10 ml of stromal medium, and the viable cell count determined by trypan blue exclusion. These cells were identified as Passage 0 (P0). The remaining cells were seeded in T225 flasks at a density of 5 x 10³ cells per sq cm. The cells were maintained in culture and passaged as described above to obtain Passage 1 (P1) ASCs, and are the cells used in this study.

4.2.2. Cryomicroscopy Experiments

The cryomicroscopy experimental procedures are similar to those described extensively in the literature [3,47,55,61,64-71]. Briefly, P1 ASCs were detached using trypsin (ATCC, Manassa, VA) and transferred to a 1.5 ml centrifuge tube, spun down for 5 minutes at 1200 rpm and re-suspended in 50µl of either 1x phosphate buffered saline (PBS) or 10% PVP (w/v) in 1x PBS solution. (It must be noted here that both the solutions contained about 1 mg/liter
concentration of a commercially available ice nucleating bacteria – pseudomonas syringae (Snomax, NY), due to the presence of which both the solutions froze spontaneously at \( \sim -10 ^\circ C \) and without which, they froze at \( \sim -18 ^\circ C \). Approximately 2.5\( \mu l \) of the sample was then transferred to the high thermal conductivity quartz crucible on the BCS-196 cryostage (Linkam Scientific™, Surrey, UK). A coverslip was then placed on top of the cell suspension within the quartz crucible to prevent leakage. During the experiments a live video signal was sent from the camera to the attached DELL personal computer and the images recorded with the help of commercially available Metacam software (Universal Imaging Corp., Buckinghamshire, UK).

### 4.2.3 The Cooling Protocols and Visualization of Intracellular Ice Formation

ASCs were cooled on the Linkam™ cryostage using two different temperature/time cooling protocols. Both the cooling protocols had a common cooling ramp: cells were cooled from 20 °C to \(-8 ^\circ C \) at 20 °C/min and then further cooled to –13 °C at 1 °C/min. No external seeding agents (e.g. chilled needle and/or ice nucleating agents) were used to initiate extra-cellular ice nucleation. At this point we employed either, cooling protocol 1: the cells were cooled from –13 °C to –40 °C at a pre-determined cooling rate of 1, 5, 10, 20 or 40 °C/min and then thawed back to 20 °C at 20 °C/min; or cooling protocol 2: the cells were re-warmed from –13 °C to –5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20 or 40 °C/min to –40 °C. The cells were then thawed back to 20 °C at a heating rate of 20 °C/min. During cooling the formation of extra- and intra-cellular ice was visually observed by the associated changes in crystalline structure and the darkening/flashing of cells, respectively [67–71]. The following table (table 4.1) comprehensively describes the cooling protocols used and the purposes for the various ramps. The first step of the common ramp was the cooling to - 8°C. This was done at a rather rapid rate of 20 °C/min so as to minimize time for the protocol.
Table 4.1. Table of Cooling Protocols: Shows the description of the steps involved in the different cooling protocols used and their purposes.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Temperature range (°C)</th>
<th>Cooling rate (°C/min)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Ramp</td>
<td>20°C to -8°C</td>
<td>20</td>
<td>for cooling specimen below freezing point</td>
</tr>
<tr>
<td></td>
<td>-8°C to -13°C</td>
<td>1</td>
<td>For visualizing phase change and growth of ice front and IIF of cells</td>
</tr>
<tr>
<td>Protocol 1</td>
<td>-13°C to -40°C</td>
<td>1, 5, 10, 20, 40</td>
<td>For observing effects of cooling rate on IIF percentage</td>
</tr>
<tr>
<td></td>
<td>-40°C to 20°C</td>
<td>20</td>
<td>For thawing back cells to room temperature</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>-13°C to -5°C</td>
<td>20</td>
<td>For thawing intracellular ice and partial thawing of extra-cellular ice</td>
</tr>
<tr>
<td></td>
<td>-5°C to -40°C</td>
<td>1, 5, 10, 20, 40</td>
<td>For observing effects of cooling rate on IIF percentage on cells that are re-cooled</td>
</tr>
<tr>
<td></td>
<td>-40°C to 20°C</td>
<td>20</td>
<td>For thawing back cells to room temperature</td>
</tr>
</tbody>
</table>

In the next ramp, the cooling velocity was slowed down to 1 °C/min so as to properly visualize and capture images during the formation of external ice. After reaching -13 °C, any one of the two protocols – cooling protocol 1 or cooling protocol 2 was employed.

4.3 Results and Discussion

4.3.1 Extra- and Intra-cellular Ice Formation (with and without PVP)

The extra-cellular solution remained unfrozen and super-cooled when cooled from +20°C to −8 °C at a cooling rate of 20 °C/min, i.e. no extra-cellular ice formed during the initial part of the common cooling ramp. However, the extra-cellular ice was formed during the second part of the common cooling ramp between −10 °C and −11 °C, as shown in Fig. 4.1. The extra-cellular ice
Figure 4.1 Intra- and Extra-cellular Ice Formation in the Common Ramp: These are images taken during the common ramp showing extra-cellular IIF (between -10 and -11°C). A & B are samples with 1x PBS whereas C & D contains 10% PVP. As can be seen from the images, as soon as extra-cellular ice starts to form, some of the cells start to undergo IIF along with it and by -13°C (refer to Fig. 4 A, B, E and F) nearly all cells are frozen.
being cooled @ 1°C/min

being cooled @ 40°C/min

Figure 4.2 (A-D) IIF in Protocol 1 (without PVP): These are representative images taken during protocol 1 (-13 to -40°C) in 1x PBS only. They show the cells undergoing IIF in this range when cooled at different cooling rates. The cooling rates used for A and C is 1°C/min while that for B and D is 40°C/min. Comparing the corresponding figures at -13 and -40°C it can be seen there is no significant difference in IIF percentage. This is due to the fact that most cells were already frozen at -13°C (the end of the common ramp and the beginning of protocol 1 or 2).
Figure 4.2 (E-H) IIF in Protocol 1 (with PVP): These are representative images taken during protocol 1 (-13 to -40°C) with 10% PVP in 1x PBS. They show the cells undergoing IIF in this range when cooled at different cooling rates. The cooling rates used for E and G is 1°C/min while that for F and H is 40°C/min. Comparing the corresponding figures at -13 and -40°C it can be seen there is no significant difference in IIF percentage. This is due to the fact that most cells were already frozen at -13°C (the end of the common ramp and the beginning of protocol 1 or 2).
nucleation was rapid and was accompanied by formation of intracellular ice in almost all (96%) of the cells between –10 and –13 °C as observed by “darkening/flashing”. Thus, at the end of the common cooling ramp, the extra-cellular medium was completely frozen and nearly all the cells had undergone IIF.

4.3.2 Cooling Protocol 1 (with and without PVP)

To reiterate, this protocol consisted of cooling the cells from –13 °C to –40 °C at a pre-determined cooling rate of 1, 5, 10, 20 or 40 °C/min (Figure 4.2). Since, the extra- and intra-cellular ice formation had already occurred, as described above in the common cooling ramp, no further changes in the extra- or intra-cellular space were observed at any of the cooling rates investigated (comparing the two rows in Fig. 4.2, A - D and E - H). Consequently, the percentage of cells exhibiting IIF remained independent of the imposed cooling rate, i.e. no statistically significant changes in the percentage of cells exhibiting IIF was noted between the various cooling rates. Thus, at the end of the experiments with cooling protocol 1, the percentage of cells exhibiting IIF was > 96% (i.e., same as that observed at the end of the common cooling ramp). Specifically, the percentage of cells exhibiting IIF at various cooling rates in PBS alone and frozen using cooling protocol 1, are 93.75%, 94.2%, 98.4%, 100%, and 100% (fig.11) for cells cooled at 1, 5, 10, 20 and 40 °C/min, from –13 °C to –40 °C, respectively. Similarly, the corresponding percentages for ASCs frozen in the presence of 10% PVP in PBS and frozen using cooling protocol 1, (fig. 12) are 96.9%, 100%, 98%, 100%, and 100% for cells cooled at 1, 5, 10, 20 and 40 °C/min, from –13 °C to –40 °C, respectively.
Figure 4.3 Freezing with Protocol 2 (without PVP): These are representative images taken during protocol 2 (-13 to -5 to -40°C) with 1x PBS as extracellular solution. They show the number of cells undergoing IIF in this range when thawed to -5°C and then cooled at different cooling rates. The cooling rates used in the cooling ramp for A through D is 1°C/min while that for E through H is 40°C/min. It can be seen that there is negligible IIF even at 40°C/min cooling rate presumably because nearly all the cells which experienced IIF at -13°C also experienced membrane damage (refer to Figure 4.8).
Figure 4.4 Freezing with Protocol 2 (with PVP): These are representative images taken during protocol 2 (-13 to -5 to -40°C) with 10% PVP as extracellular solution. They show the number of cells undergoing IIF in this range when thawed to -5°C and then cooled at different cooling rates. The cooling rates used in the cooling ramp for A through D is 1°C/min while that for E through H is 40°C/min. With 10% PVP in 1x PBS, it can be seen that once the cells are thawed and re-frozen, they do not experience IIF with 1°C/min but do so again with 40°C/min.
4.3.3 Cooling Protocol 2 (in 1x PBS and without PVP)

To reiterate, this protocol consisted of re-warming the cells from –13 °C to –5 °C at 20 °C/min and then the cells were re-cooled at a pre-determined rate of 1, 5, 10, 20 or 40 °C/min to –40 °C (Figure 4.3). During the re-warming of the samples from –13 °C to –5 °C, the extra- and intra-cellular space was partially thawed and the ice formed within the cells was transformed into an transparent phase (as opposed to the opaque/dark phase), i.e., most probably due to the fact that the ice within the cells melted. It is to be noted that the phase change temperature of 1x PBS, based on the well established formula for melting point of solutions, $T_m$ (melting point) = 273.15 – 1.858*Osm, K, where Osm is the osmolality of the solution, was found to be 272.62 K or -0.5 °C (the osmolality of the 1x PBS solution as measured by a Wescor™ vapor pressure osmometer was 287 ± 4 mOsm). However, further cooling of these cells from –5 °C to –40 °C at various cooling rates (1, 5, 10, 20 and 40 °C/min) did not result in re-nucleation or “darkening/flashing” in any of the cells, i.e. none of the frozen-thawed cells exhibited IIF. Specifically, the percentage of cells exhibiting IIF at various cooling rates in PBS alone and frozen using cooling protocol 2, are 4.6%, 3.2%, 3.9%, 0%, and 4.6% for cells cooled at 1, 5, 10, 20 and 40 °C/min, from –5 °C to –40 °C, respectively (fig. 4.5).

4.3.4 Cooling Protocol 2 (with 10% PVP in 1x PBS)

To reiterate, this protocol consisted of re-warming the cells from –13 °C to –5 °C at 20 °C/min and then the cells were re-cooled at a pre-determined rate of 1, 5, 10, 20 or 40 °C/min to –40 °C (Fig. 4.4). As before, during the re-warming of the cells/solution from –13 °C to –5 °C, the extra- and intra-cellular space was partially thawed and the ice formed within the cells was transformed into an transparent phase (as opposed to the opaque/dark phase). Note that the phase change temperature of the extra-cellular solution based on the osmolality is nearly -1°C
During further cooling, i.e. cooling from –5 °C to –40 °C, several phenomena were observed, namely: i) trace amount of ice crystals present in the extra-cellular medium, acted as nucleating agents; ii) consequently, the ice crystals formed were much larger compared to those obtained during extra-cellular ice nucleation in the common cooling ramp and/or cooling protocol 1. This is illustrated by a comparison of the images shown in fig. 10. This result is not surprising as it is well known that the size of ice crystals formed during nucleation is inversely correlated with the amount of supercooling [3,47,55,56,64-71,73]. Most importantly, further cooling of these cells from –5 °C to –40 °C at various cooling rates (1, 5, 10, 20 and 40 °C/min) did result in re-nucleation or “darkening/flashing” in most of the cells, i.e. most of the frozen-thawed cells exhibited IIF. Specifically, 0%, ~40%, ~47%, ~67% and ~100% (fig. 4.6) of the ASCs frozen in 10% PVP in PBS exhibited re-nucleation or “darkening/flashing” when cooled at 1, 5, 10, 20 and 40 °C/min, from –5 °C to –40 °C, respectively. The observed difference in the percentage of cells exhibiting IIF at different cooling rates can be attributed to the intrinsic statistical dependence of the formation of intracellular ice on the imposed cooling rate [3,47,55,56,64-71]. Basically, the imposed cooling rate of 1˚C/min was sufficiently “slow” to avoid IIF and hence, the all of the cells experienced water transport or cellular dehydration at this cooling rate. As the cooling rate is increased the fraction of cells experiencing IIF increases and correspondingly, the fraction of cells experiencing water transport decreases. And at the “fast” rate of 40 °C/min all the cells exhibit IIF. And finally, the fraction of cells undergoing IIF as a function of sub-zero temperature and the imposed cooling rate, in the presence of PVP and cooled using protocol 2, is shown in Figure 4.7.
Figure 4.5 Percentage IIF Comparison (without PVP): Shows the comparative values of IIF percentage at -40°C between protocol 1 and 2, for cells frozen in 1x PBS. The numbers in brackets shows the total number of cells that underwent IIF out of the total number of cells that were frozen. For protocol 1, these numbers are not significantly different from the numbers at the end of the common ramp proving that the freezing rate (1,5,10,20, 40°C/min) in protocol 1 have very little effect on their values. As can be seen, the thawing and re-freezing ramps with protocol 2 result in negligible IIF in 1x PBS, presumably as most of the cells were already damaged during their IIF at the end of the common ramp (refer Fig. 4.8).

Figure 4.6 Percentage IIF Comparison (with PVP): Shows the comparative values of IIF percentage at -40°C between protocol 1 and 2, for cells frozen in 10% PVP. The numbers in brackets shows the total number of cells that underwent IIF out of the total number of cells that were frozen. For protocol 1, these numbers are not significantly different from the numbers at the end of the common ramp proving that the freezing rate (1,5,10,20, 40°C/min) with protocol 1 have very little effect on their values. However, the thawing and re-freezing ramps with protocol 2 result in varying percentages of IIF.
4.3.5 Fluorescent Dye Exclusion Tests

As mentioned in section 4.4.2., when the cells were frozen according to cooling protocol 1 in 1x PBS (without PVP), no significant re-nucleation of intracellular ice was observed with any of the 5 pre-determined cooling rates. To further investigate this phenomena, i.e., lack of IIF in the frozen-thawed cells, we measured the ability of these cells to exclude a fluorescent dye. Briefly, the cells at the end of the initial cooling step in cooling protocol 2, i.e., the cells thawed from $-13\,^\circ\text{C}$ to $-5\,^\circ\text{C}$ were further thawed to $20\,^\circ\text{C}$ at $20\,^\circ\text{C/min}$. The viability of these cells was assessed using propidium iodide (PI) dye exclusion, as previously described [42]. Briefly, the P1 ASCs were suspended in 1x PBS as described in section 4.2.2. However, this time the 1x PBS solution also contained 1.5 µg/ml of propidium iodide. Approximately all of the cells (>90%) were unable to exclude the dye and hence, had compromised membrane integrity (fig. 4.8). This suggests that the formation of intracellular ice during the common cooling ramp, between $-10$ and $-13\,^\circ\text{C}$, was extremely deleterious and damaging. Hence, these cells with
compromised membranes did not and could not exhibit IIF on further re-cooling and further, exhibited morphological distortions consistent with compromised membrane integrity (Figs. 9C, 9D, 9G and 9H). Similar tests were also carried out with cells suspended in 10% PVP in 1x PBS. Based on our earlier observations of these cells to re-nucleate and exhibit IIF, we fully expected that these viability numbers will be significantly higher than that obtained for cells frozen in the absence of PVP. Indeed, this was found to be the case (data not shown). This led us to the inference that the presence of PVP has a significant role in protecting the cells during the appearance of intracellular ice that accompanies extra-cellular ice nucleation in the common ramp.

The equilibrium melting point of 1x PBS is ~ –0.6 °C and that of 10% PVP in 1x PBS is ~ –1°C [72]. However, the use of a finite cooling rate instead of infinitesimally small cooling rate coupled with the lack of external seeding e.g. with a chilled needle, the solutions exhibit super-cooling to temperatures as low as –10 °C. Obviously, once nucleation occurs in such a highly unstable and super-cooled medium, the process of ice formation is very rapid and ice freeze-front covers the field of view almost instantaneously [48,66,67,70-72]. It has been postulated that the probability of ice formation is significantly increased if the extra-cellular medium freezes after a high degree of super-cooling or at a low sub-zero temperature, mainly because of two reasons: i) the high degree of cooling rates experienced by the sample due to the thermal fluctuations caused by the release of latent heat of the extra-cellular medium and ii) due to the high amount of intracellular super-cooled water retained within the cells due to the lack of cellular dehydration [3,47,64-66,69-71]. Thus, during the common cooling ramp, a significant % of the cells exhibit IIF; specifically 1015 cells exhibited IIF out of a total 1060 cells investigated as part of this study (or ~96%).
Figure 4.8 Propidium Iodide Exclusion Tests: Shows the results of the propidium iodide exclusion tests with 1x PBS and 1.5 µg/ml propidium iodide (A & B). Cells were made to cool through the common ramp till -13°C (where they experienced IIF as before) and then thawed back to room temperature. Figure A shows the number of dead cells (~2) out of ~187 cells at start and B shows the number of dead cells at the end of the experiment (~170).
5. CONCLUSION AND FUTURE WORK

The main objective of this work was to assess the effect of 10% (w/v) PVP on the pattern of IIF in ASCs in the absence of serum and other cryoprotective agents (CPAs). All of the cells were cooled in a common cooling ramp from 20 °C to –8 °C at 20 °C/min and then further cooled to –13 °C at 1 °C/min (during which the extra-cellular medium froze very rapidly and was accompanied by the formation of intra-cellular ice in ~90% of the cells). Almost none (<5%) of the ASCs frozen in 1x PBS and protocol 2 (re-warmed from –13 °C to –5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20 or 40 °C/min to –40 °C) exhibited IIF. The lack of IIF in cells cooled in 1x PBS and protocol 2 was due to the initial loss of cell viability that was associated with the formation of extra-cellular ice and associated IIF in the common cooling ramp. Intriguingly, ~0, ~40, ~47, ~67 and ~100% of the ASCs frozen in 10% PVP in PBS and protocol 2 exhibited IIF at a cooling rate of 1, 5, 10, 20 or 40 °C/min, respectively. The observed increase in the percentage of ASCs exhibiting IIF when frozen in 10% PVP and protocol 2, is presumably due to PVP mitigating the damaging effects of IIF during the initial common cooling ramp. The results of this study can be hence used to construct cryopreservation protocols for ASCs without the use of serum and DMSO.

The optimal cooling rate that is required for the cryopreservation of ASCs without serum and DMSO to produce the maximum post thaw viability still remains to be ascertained. Future researchers may want to look into this data and use cooling protocol 2 and 10% PVP and use “flow-cytometry analysis” as used in the work by Thirumala et al [26] to find out more accurate viability numbers for this process. It is probable that the cooling method used in the work by Thirumala et al [26] (freezing ASCs over-night in an ethanol-jacketed container in a –80°C freezer) suffers from the drawback that the intracellular ice formed in the cells during nucleation
of the extracellular medium is not melted (as in the thawing ramp of protocol 2) and hence cannot lose enough water to avoid IIF injuries at further lower temperatures. The fact that 0% IIF with 1 °C/min and ~ 40% IIF with 5 °C/min cooling rate was observed in the re-freezing step of protocol 2 with an extracellular medium of 10% PVP, hints to the existence of a optimum cooling rate within a narrow window which can be found out with further freezing studies. It should also be noted that the standard equations of water transport (equations 6 and 7) do not apply to this particular system containing PVP in the extracellular medium. This is because of the fact that the Mazur’s equations of water transport have been derived on the basis of the assumption that both the intra- and the extra-cellular solutions are ideal solutions following Raoult’s Law. However, in this case, the extra-cellular solution containing PVP deviates significantly from ideal behavior of solutions and does not follow Raoult’s law of ideal solutions. The non-ideal behavior of PVP has been shown elsewhere [74] and the water transport equations of such a system with the extra-cellular medium containing a polymer (PVP), salts and water needs to be suitably modified by using volume fractions instead of mole fractions and the interaction parameter ($\chi$) of PVP-water.
REFERENCES


VITA

Avishek Guha was born in Calcutta, West Bengal in India in June, 1980. He received his primary and secondary education from South Point High School, Calcutta. After graduating from high school in 1999, he pursued Bachelor of Engineering degree in Jadavpur University, Calcutta. In 2003, he graduated from Jadavpur University and joined TVS Motor Company in Hosur, India as a graduate engineering trainee where he worked for one year in fabrication division. He then shifted to HV Transmissions Ltd. (Tata Motors) in Jamshedpur, India and worked there for three years till July, 2007 in Ancillary Development Department. In the fall of 2007 he went to graduate school in Louisiana State University. He is a candidate for the degree of Master of Science in Mechanical Engineering to be awarded at the commencement of December, 2009. After completion of the degree, he intends to join the doctoral program in mechanical engineering at Louisiana State University from spring of 2010.