University of Alberta

Using Simulations to Design a Cryopreservation Procedure for Hematopoietic Stem Cells Without DMSO

By

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Abstract

The purpose of this study was to utilize simulations based on cellular osmotic properties to design a cryopreservation protocol. These simulations were used to minimize the amount of damage incurred by the cells during the freezing process by optimizing the cooling conditions. There is significant interest in designing a cryopreservation protocol for hematopoietic stem cells which does not rely on dimethyl sulfoxide (DMSO) as a cryoprotectant, since toxicities have been associated with infusion of stem cells cryopreserved with DMSO. The TF-1 cell line was used as a model system to analyze cellular responses to low temperature. Osmotic parameters were determined and used to simulate the cellular responses without cryoprotectant to various cooling conditions, such as linear and non-linear cooling rates, subzero temperatures and hold times. These simulations were then used to predict an optimal cryopreservation protocol. The protocol was validated empirically using a graded freezing and a 2-step freezing protocol.

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Abbreviations

NaCl	sodium chloride
DMSO	dimethyl sulfoxide
HES	hydroxyethyl starch
HSC	hematopoietic stem cells
CD34	cluster designation 34
FBS	fetal bovine serum
GM-CSF	granulocyte macrophage-colony stimulating factor
PBS	phosphate-buffered saline
k	fiiting constant
CO_2	carbon dioxide
H_2O	water
KCl	potassium chloride
EB	ethidium bromide
UV	ultraviolet
DNA	deoxyribonucleic acid

Symbols

L_p	hydraulic conductivity
v_b	osmotically-inactive fraction
E_a	Arrhenius activation energy
V	cell volume

t	time
A	cell surface area
R	universal gas constant
T	absolute temperature
π	osmolality
V_{eq}	equilibrium volume
V_{iso}	isotonic volume
[ ]	concentration

Subscripts and Superscripts

e	extracellular
i	intracellular
o	isotonic

Chapter 1: Introduction

1.1 Cryobiology

Cryobiology is the study of the effects of low temperatures on biological systems. Although freezing is lethal to most living systems, cryobiologists have been able to preserve cells and tissues at a range of subzero temperatures, as low as liquid nitrogen temperatures (-196°C). Currently, cryopreservation is limited to most cells in suspension; such as hematopoietic stem cells, red blood cells, other progenitor cells, and sperm. Cryopreservation has also been used to effectively preserve some tissues, such as heart valves, embryos, blood, skin, articular cartilage, and islet of Langerhans. Although the recovery of viable cells post-thaw is sufficient for clinical use, it is generally considered less than optimal due to injury during the freezing process.

During cryopreservation, a cell is slowly cooled to subzero temperatures, where ice is formed extracellularly, leaving the intracellular water unfrozen. This unfrozen fraction is supercooled below its freezing point. Intracellular supercooling is the extent to which a cell is cooled below the phase-change temperature before the formation of ice inside the cell (intracellular ice). As water is removed by the formation of ice in the extracellular environment, the concentration of extracellular solutes is increased [22]. There is a resultant increase in intracellular solute concentration as well, as water flows out of the cell by ex-osmosis or through aquaporins (water channels). The cell can only maintain equilibrium with the extracellular solution if the cooling rate is slow

enough that the cell can dehydrate at the same rate as ice is forming. However, if the cooling rate is too rapid for the cell to lose water to the extracellular environment, the intracellular water becomes increasingly supercooled and there is increased probability of the formation of intracellular ice.

There have been many theories proposed to explain the mechanisms of freezing injury. Mazur proposed that during rapid cooling, cell injury is caused by the formation of intracellular ice. The formation of intracellular ice can be caused by supercooled intracellular water, as cells have insufficient time to equilibrate with the extracellular compartment by dehydration. The response of cells to the formation of ice in the surrounding solution is largely dependent on the movement of water across the plasma membrane [28]. Cell membrane permeability limits water efflux at low temperatures, resulting in supercooling at high cooling rates. Mazur also proposed that there is a 10°C limit to supercooling, above which the probability of the formation of intracellular ice is significantly increased. The probability of intracellular ice increases with the amount of supercooling [28].

Lovelock also proposed that during the freezing process, cells are subjected to high concentrations of sodium chloride (NaCl) due to the formation of ice in the extracellular compartment [25]. As ice is formed, water is removed from the solution as ice, resulting in increased concentrations of NaCl in the liquid phase. This results in osmotic stress on the cell, which causes water to leave the cell. The dehydration of the cell also causes increased concentration of salts inside the cell. Lovelock proposed that the increasingly high

concentration of solutes both extracellularly and intracellularly is damaging to the cell as the cell membrane becomes leaky. Meryman later proposed that it is not a high concentration of salt that is damaging but that there is a minimum tolerable cell volume due to cell shrinkage during the dehydration process that is damaging to the cell [37]. Therefore, there is a minimum limit to the cell volume, which, regardless of the osmotic stress imposed by the environment, the cell cannot shrink beyond. This osmotic stress results in a transient pressure gradient across the membrane, which in turn causes the membrane to become leaky [29].

Mazur et al.'s 'two-factor hypothesis', combined previous works on the types of freezing injury and proposed that there are two independent mechanisms of damage during freezing: slow cooling, where cell injury is due to exposure to high solute concentrations due to ice formation, and rapid cooling, where cell injury is caused by the formation of intracellular ice [32]. Maximum recovery is obtained by minimizing solution effects when cells are cooled rapidly enough to avoid harmful effects of their environment, yet cooling cells slowly enough that the cells can dehydrate sufficiently to avoid intracellular ice formation.

There is also evidence suggesting that damage may not be restricted to injury during freezing. The small ice crystals formed during rapid cooling may recrystallize during warming, causing additional damage to the cell [12,30-32]. The resulting damage occurs primarily during slow warming and may be more damaging than the formation of ice itself [12,30-32]. Therefore, there exists an

optimal cooling and warming rate specific for each cell type, which minimizes both types of injury.

Injury sustained during a cryopreservation procedure can be further minimized by the use of cryoprotectants. Cryoprotectants are classified, as permeating and non-permeating, based on their ability to traverse the cell membrane [36]. Penetrating agents, such as dimethyl sulfoxide (DMSO), protects the cell against slow cool injury by reducing the amount of ice formed, thereby minimizing cell dehydration [36]. Glycerol, has also been shown to protect the cell when present both inside and outside the cell, by reducing the excessive concentration of salts [26,38]. The criteria for an effective penetrating cryoprotectant are that it must penetrate the cell and exhibit low-toxicity at multimolar concentrations [36]. DMSO protects the cell by depressing the freezing point, which lowers the temperature at which ice is formed and in turn lowers the temperature where a specific concentration of electrolytes occurs [4]. The movement of penetrating cryoprotectants is also dependent on the membrane permeability and is limited at low temperatures. Non-penetrating cryoprotectants are high molecular weight additives, such as starches and sugars, which do not permeate the cell membrane. These agents confer protection by promoting water loss at higher subzero temperatures and are responsible for the osmotic stress on the cell [4]. McGann reported that freezing Chinese hamster fibroblasts with 20% hydroxyethyl starch (HES), resulted in comparable cell recovery to using 10% DMSO/media [33].

In cryopreservation procedures, cells are cooled at a finite rate which is

optimized for the cell type and cryoprotectant. This optimization has typically been approached empirically by varying cooling rates and the cryoprotectants. In addition to cooling at a constant rate, there are two other techniques which have been used to examine the effects of low temperatures on cells: a two-step freezing technique and a graded freezing technique. The two-step freezing technique, used by Farrant et al. is a logical method to examine the effects of osmotic interactions on cell recovery over a broad range of subzero temperatures [11]. In this procedure, lymphocytes were cooled rapidly to various subzero temperatures and held for various periods of time before being 1) thawed directly from that holding temperature or 2) rapidly cooled to -196°C before thawing. McGann and Farrant later reported the subzero temperature and the length of hold time at that temperature were important factors to consider when attempting to maximize cell survival [35]. The graded freezing technique was later developed by McGann and used to determine the temperature range through which slow cooling should be controlled [34]. Samples were cooled slowly to various subzero temperatures before being either thawed directly or plunged into liquid nitrogen first and then thawed. These experimental techniques provided insights into the effects of subzero temperatures and time, which can be used to empirically optimize a cryopreservation procedure.

Minimizing the potential for solution effects injury and injury due to intracellular ice formation can be done by controlling the water content in the cell. Movement of water due to extracellular ice formation ultimately determines cell volume. However, the movement of the cryoprotectant also contributes to the

changes in cell volume. Therefore, it is the interactions of water and solute movement which are important factors in cellular responses to freezing.

1.2 Cell membrane permeability

Water movement across the semi-permeable plasma membrane occurs due to changes in the extracellular osmolality. In a hypertonic environment, a cell will dehydrate to reach a new equilibrium with the environment, with a resultant reduction in cell volume. Conversely, in a hypotonic environment, a cell will swell, with a resultant increase in cell volume. The permeability characteristics of the cell membrane control the movement of water both into and out of the cell. There are changes in the fluidity of the membrane that occur at lower temperatures so water movement is reduced [10,24]. The cellular responses to the changes in osmolality parallel the changes in cell volume as ice is formed in the extracellular environment and are thus important in cryobiology [29].

The osmotic characteristics of the cell membrane, which govern the movement of water, are the hydraulic conductivity, the osmotically-inactive fraction and the Arrhenius activation energy. The hydraulic conductivity (L_p) is the rate at which water permeates the cell membrane. Jacobs and Stewart described L_p using the rate of volume change in anisotonic solutions at an experimental temperature [16]. Using this description, L_p can be determined using measurements of the kinetics of volume change over time when exposed to anisotonic solutions. The osmotically-inactive fraction (v_b) of the cell is the fraction of the cell volume not involved in the osmotic activities of the cell. The

Boyle van't Hoff relationship can be used to calculate the osmotically-inactive fraction by expressing the equilibrium cell volume after exposure to anisotonic solutions of impermeant solutes. The Arrhenius activation energy (E_a) for L_p is normally used to describe the temperature dependence of the L_p. E_a can be determined using the slope of the Arrhenius plot of the natural logarithm of L_p as a function of the inverse absolute temperature (K) [42]. At lower temperatures, there are changes to the cell membrane, such as the lipid bilayer membrane may undergo phase transitions to a gel-like state, so water movement is reduced [42]. Therefore it is important to determine the temperature dependence of the osmotic parameters in order to explore the cellular responses at low temperatures. Cellular responses to freezing and thawing can be described using osmotic parameters to conduct simulations.

1.3 Simulations of low temperature responses

Computer simulations of cellular responses to low temperatures are based on mathematical calculations of changes in cell volume. It is possible to use the osmotic parameters to calculate cellular responses, ie. changes in cell volume. Modeling expresses the theoretical cellular responses to anisotonic environments based on the osmotic properties of the cell membrane and their temperature dependencies which are specific to each cell type. These simulations can be extrapolated to low temperature responses due to changes in osmolality by the formation of ice. The formation of ice imposes osmotic stresses on the cell by the concomitant increase in concentration of extracellular solutes [9]. Thus, the permeability characteristics of the cell membrane regulate the intracellular

osmolarity and, in turn, cell volume.

Simulations are based upon the phase diagrams of extracellular and intracellular solutions, the membrane permeability characteristics of the cell and the temperature dependence of the osmotic parameters. The phase diagrams describe the composition of the solutions during the freezing process and can be used to calculate the changes in cell volume based on the concentration of electrolytes in the unfrozen fraction of the system. Furthermore, different initial concentrations of electrolytes in the extracellular and intracellular solutions can be used in simulations to calculate the increase in final concentrations during freezing and to determine the potential for injury due to exposure to electrolytes. The osmotic characteristics of the cell membrane can be used to predict the cellular responses to low temperatures, as the movement of water across the membrane is governed by the hydraulic conductivity, which is temperature dependent. Simulations of freezing protocols attempt to minimize freezing injury theoretically, based on the membrane characteristics of the cell, as opposed to empirically, based on cooling rates and the addition of cryoprotectants.

Traditionally, cryopreservation protocols have been determined empirically, but some researchers have explored the use of simulations as a means to predict low temperature responses of various cell types: bovine erythrocytes [22]; yeast [40]; hamster ova [41]; hamster pancreatic islet cells [24]; and epithelial, endothelial and stromal cells [8]. Simulations can be used to predict the potential for intracellular ice formation based on the amount of supercooling. Mazur proposed that there was a 10°C limit to supercooling prior

to the formation of intracellular ice and the risk of intracellular ice increased based on the amount of supercooling [28]. Therefore the likelihood of intracellular ice formation is significant if the supercooling exceeds 10°C. In yeast cells, low cooling rates have been shown to reduce the amount of supercooling and thus reduce the risk of intracellular ice [28]. Diller further examined the probability of intracellular ice formation based on the interaction of cooling rates and supercooling on human erythrocytes [5]. During freezing, the cell attempts to maintain equilibrium across the plasma membrane either through osmotic dehydration and/or the formation of intracellular ice [5]. Sub-optimal or supra-optimal cooling rates will cause damage to a cell either by solution effects or intracellular ice formation, respectively. Ebertz et al. also demonstrated the use of simulations for cryopreserving corneal cells by examining supercooling as a function of cooling rates to reduce the amount of experimentation required and define a starting point for experimental verification of predicted optimal cooling rates [7,8]. This thesis research will further the use of simulations using *CryoSim5* (Dr. Locksley McGann, University of Alberta, Canada), to design a cryopreservation protocol for hematopoietic stem cells.

1.4 Cryobiology of Hematopoietic Stem Cells

Cryobiology has been applied to many cell and tissue types. Recent developments in the utilization of a variety of stem cells, including cord blood stem cells have revived interest in optimizing cryopreservation techniques for hematopoietic stem cells (HSC). HSCs are capable of both self-renewal and differentiation into all mature hematopoietic blood cells [21]. Cryopreservation of

cord blood stem cells and other cell types depend critically on the low number of cells obtained from each donor and high recovery of these cells is essential. This is also applicable to the cryopreservation of engineered cells due to the high cost and length of time for manufacturing cells. Higher standards for cell banking, specifically HSC banking, are required to meet future needs of cell banking, and therefore optimal cryopreservation techniques are fundamental.

HSC transplantation has been used to successfully treat a broad spectrum of malignant and non-malignant diseases. Sources of HSCs include bone marrow, peripheral blood, and umbilical cord blood. HSCs represent a very small subset of hematopoietic cells with the capacity to self-renew and differentiate, thus providing a constant supply of the cells of the entire hematopoietic spectrum. The generation of mature blood cells from HSCs involves a highly regulated progression through successive stages of commitment to a specific cell lineage. The expression of different receptors on the surface of HSCs permits the interaction with various regulatory elements. CD34 has been used as a convenient marker for HSCs, since $CD34^+$ cells have been shown to possess colony-forming potential and allow the expression and differentiation of blood cells from different hematopoietic lineages [13].

The growing use of high-dose chemotherapy followed by stem cell transplantation requires efficient HSC collection, cryopreservation and storage. Reconstitution of hematopoiesis after myeloablation with high-dose chemotherapy is dependent on the number, as well as the quality of HSCs. Cryoprotectants can be used to maximize cell post-thaw recovery. Currently,

cryopreservation of these stem cells has been most successful with the use of DMSO. There are, however, limitations to the use of DMSO. Toxicities have been associated with infusion of stem cells preserved with DMSO [4,9,39,43]. For cord blood samples, high cell recovery is critical because of the small, limited sample volume. There is significant interest in designing a cryopreservation protocol for HSCs, which limits toxicity, as well as maintains the cell viability while not requiring DMSO as a cryoprotectant. Some researchers have attempted to reduce the amount of DMSO [1,2] or combine it with a non-penetrating cryoprotectant, such as Hydroxyethyl starch (HES) [6,14,17].

It has also been shown that cooling and warming rates are also important factors in efficient cryopreservation of HSC [36]. For HSCs, it has been shown, using a 10% DMSO solution, that cooling rates of 1°C/min can lead to a high recovery of cells [15] and this is used universally as a protocol for cryopreserving HSCs. It has also been shown that a cooling rate of 3°C/min results in a higher cell recovery [36]. However few HSC banking groups have adopted this protocol. Also, Leibo et al. reported the deleterious effects of slow warming on HSC both with low cooling and high cooling rates [23], which was later supported by McGann et al. [36]. Both studies demonstrated that rapid warming at 37°C resulted in the highest cell recovery. Optimizing the cryoprotectant solution and the cooling and warming rates is essential in determining the optimal freezing protocol for HSC. This study uses TF-1 cells as a model cell type for HSCs. Derived from a erythroleukemic patient, TF-1 cells express the $CD34^+$ antigen and are able to differentiate into the various hematopoietic lineages [18,19,27].

TF-1 cells have been used as a tissue culture model for various HSC functions, such as JAK/STAT pathway mechanisms of activation [20], CD43 induced apoptosis [3], and TGF-β1 induced differentiation [27]. Although, ultimately we propose the use of simulations as a tool to cryopreserve patient HSC samples, tissue culture cells provide more consistent and repeatable results needed to validate simulations with empirical data.

1.5 Hypotheses

1. Simulations, based on osmotic parameters, can be used to predict cellular low temperatures responses.

2. Simulations can be used to design and optimize cryopreservation protocols for a HSC experimental model.

1.6 Objectives

Traditionally, cryopreservation of cells has been approached empirically. This thesis presents the use of theoretical modeling, as opposed to purely empirical experimentation, as an approach to the development of cryopreservation protocols. The objectives are:

1. Determine the osmotic parameters, hydraulic conductivity, osmotically-inactive fraction, and Arrhenius activation energy, of TF-1 cells.

In Chapter 2, we measured the osmotic parameters using an electronic particle counter fitted with a cell size analyzer. Changes in cell volume as a function of time were monitored, while exposing cells to hypertonic

solutions at various temperatures.

2. Utilize the osmotic parameters of TF-1 cells to model the cellular responses to low temperatures in the graded freezing procedure.

In Chapter 3, we used these osmotic parameters of TF-1 cells to model the cellular responses to low temperatures. These models were based on actual experimental procedures, ie. graded freezing protocols, which have been used to examine the effects of high solute concentrations and intracellular ice formation. The results of the simulations were then used to predict the optimal procedure for cryopreserving TF-1 cells.

3. Empirically test the simulations using graded freezing experiments to compare with theoretical data.

In Chapter 4, we used graded freezing of TF-1 cells to document cryoinjury and the results were compared with the theoretical predictions of Chapter 3.

4. Simulate a two-step freezing procedure as a novel approach to cryopreservation of cells without DMSO.

In Chapter 5, we used the osmotic parameters of TF-1 cells to model cellular responses to low temperatures based on a two-step freezing protocol, which has been used to examine the effects of subzero temperatures and time spent at subzero temperatures on the two types of

freezing injuries: solution effects and intracellular ice formation. The results were then used to design and optimize a practical protocol for cryopreserving TF-1 cells.

5. Empirically test and optimize the designed protocol.

In Chapter 6, we used a two-step freezing procedure to document cryoinjury and the results were compared with the theoretical protocol designed using simulations of Chapter 5.

Defining the osmotic responses of TF-1 to anisotonic solutions, modeling cellular responses to low temperatures and validating these responses empirically are the main objectives of this thesis. The overall goal of this thesis is to develop approaches using simulations that can be applied to the optimization and design of cryopreservation procedures for HSCs and other cell types.

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Chapter 2: Determination of cellular osmotic parameters of TF-1 cells

2.1 Introduction

Osmotic responses of cells to the formation of ice in the surrounding solution are largely dependent on the movement of water across the plasma membrane [21]. The formation of extracellular ice and the resulting increase in extracellular solute concentration, impose osmotic stresses on the cell [22]. The osmotic parameters governing the movement of water across the membrane are specific to each cell type. Thus different cells respond differently to anisotonic conditions. The movement of water across the membrane is faster than the movement of solutes and is the result of simple diffusion of water molecules across the plasma membrane or the result of water movement through water channels or aquaporins. A significant amount of the cell volume is comprised of water therefore water movement determines the cell volume. The net water movement is described using the osmotic parameters of the cell membrane.

The osmotic parameters, which govern water movement, are the hydraulic conductivity, the osmotically-inactive fraction and the Arrhenius activation energy. The hydraulic conductivity (L_p) denotes water transport across the cell membrane and thus the cell volume. L_p is a function of the rate at which water moves across the cell membrane. Jacobs and Stewart [15] uses the following equation, which describes the rate of cell volume change in anisotonic solutions as a function of

L_p (μm/min/atm) :

$$\frac{dV}{dt} = L_p \cdot A \cdot R \cdot T(\pi_i - \pi_e) \quad (1)$$

where V is the cell volume (μm^3), t is the time (min), A is the cell surface area (μm^2), R is the universal gas constant (kcal/mol/K), T is the absolute temperature (K), π_e is the extracellular osmolality (osmoles) and π_i is the intracellular osmolality (osmoles). The Boyle van't Hoff relationship expresses equilibrium cell volume in solutions of impermeant solutes:

$$\frac{V_{eq}}{V_{iso}} = \frac{\pi_o}{\pi}(1 - v_b) + v_b \quad (2)$$

where V_{eq} is the equilibrium volume (μm^3), V_{iso} is the isotonic volume (μm^3), π_o is the isotonic osmolality (osmoles), π is the experimental osmolality (osmoles), and v_b is the osmotically-inactive fraction. Through graphical analysis of V_{eq}/V_{iso} as a function of π_o/π, v_b can be determined by extrapolating the line by linear regression to the y-intercept.

The movement of water across the cell membrane is temperature dependent. The Arrhenius activation energy (E_a) for L_p is normally used to describe the temperature dependence of the hydraulic conductivity [29]. E_a (kcal/mol) can be determined using the slope of the Arrhenius plot of the natural logarithm of L_p as a function of the inverse absolute temperature (K):

$$L_p = k \cdot \exp(\frac{-E_a}{R \cdot T}) \quad (3)$$

where k is a fitting constant, R is the universal gas constant (kcal/mol/K) and T is the absolute temperature (K). Osmotic parameters are useful for computer

simulations which model changes in cell volume at low temperatures and which could eventually be applied to more complex systems, such as tissues.

An electronic particle counter was used to monitor cell volume as a function of time for cells exposed to hypertonic solutions. In the past, electronic particle counters have been used for a variety of cell types: lymphocytes [12]; chondrocytes [27]; pancreatic islet cells [19,30]; human corneal endothelial, stroma, and epithelial cells [4] and selected African mammalian spermatozoa [8]. As cells pass through the aperture of the electronic particle counter a volume of conducting fluid is displaced resulting in a current pulse, which is proportional to the cell volume. In kinetic studies, sequential measurements of cell volumes allow for the determination of cell permeability characteristics by fitting the experimental data with theoretical models. An electronic particle counter allows permeability characteristics to be obtained for osmotically slow responding cells [1]. A computer interfaced to a particle counter can record the volume and time of measurements, so the time evolution of cell volume distribution can be monitored. Another technique using optical measurements has also been used to study osmotic responses of other cell types [2,7,13]. Acker et al. compared the two techniques and determined that even though there are no direct measurements of single cell volumes using an electronic particle counter, there was no significant difference in cell volume measurements between the two techniques [1]. The electronic particle counter method was used in these experiments because it provides rapid and reproducible data collection for analysis of a population of cells in one experiment, as opposed to multiple single-

cell analyses required by optical measurements.

The objective of this study was to use an electronic particle counter fitted with a cell size analyzer, to measure changes in cell volume as a function of time while exposing the cells to hypertonic solutions for TF-1 cells, as a model for hematopoietic stem cells (HSC) [16,17,20]. The osmotic parameters and temperature dependencies were then calculated from the volume measurements.

2.2 Materials & Methods

TF-1 cell culture

TF-1 cells (ATCC, Manassas, Virginia) were cultured at 37°C in 5% CO_2 in RPMI 1640 Medium Modified (ATCC) with 10% fetal bovine serum (FBS) (ATCC), and supplemented with 2 ng/mL recombinant human GM-CSF (Stemcell Technologies, Vancouver, Canada). Cells were cultured at a concentration between 0.1 x 10^6 and 1 x 10^6 cells/mL, according to ATCC guidelines. Prior to experiments, cells were washed twice with serum-free RPMI media and incubated overnight. TF-1 cells cultured in RPMI without FBS and GM-CSF overnight accumulate in the G_1/G_0 phase of the cell cycle [18], resulting in a more uniform cell size distribution. Cells were then centrifuged and re-suspended in serum-free RPMI at 4 x 10^6 cells/mL for osmotic measurements.

Experimental solutions

Various concentrations of phosphate-buffered saline (PBS) were used to examine the concentration-dependence of the hydraulic conductivity and the osmotically-inactive fraction. PBS solutions (1-5X) were made by diluting 10X PBS (GIBCO) with distilled water to final osmolalities of 291±6, 583±25, 861±22,

1150±17 and 1434±20 mOsm/kg respectively. Osmolalities were measured using a freezing-point depression Osmometer (Precision Systems Inc., Natick, Massachusetts), which was calibrated using 100, 300 and 500 mOsm/kg osmometry standards (Precision Systems Inc.).

Measurements of cell volumes

The Coulter counter (ZB1, Coulter Inc., Hialeah, Florida), fitted with a pulse-height analyzer (The Great Canadian Computer Company, Spruce Grove, AB, Canada) was used to monitor cell volume as a function of time as cells passed through the 100 μm aperture [9-11,26]. This system has been previously used to monitor changes in cell volume for a variety of cells in suspension [2-4,12,19,23,24,30,31], including hematopoietic stem cells [6,13,25,29].

TF-1 cells (150-200 μL) were injected into well-mixed hypertonic experimental solutions (10 mL). Experimental solutions were maintained at experimental temperatures using a circulating water bath with a custom insulated jacket. The current pulses proportional to the cell volumes were measured and the time recorded as the cells passed through the aperture of the Coulter counter. Experimental temperatures were measured at 4.6±0.7, 4.8±0.6, 8.1±0.7, 11.1±0.6, 12.9±1.4, 16.4±0.5, 19.4±0.8, 23.3±1.2, 28.8±0.6, and 37.4±0.8°C using a Digi-Sense thermocouple thermometer (Cole Parmer, Anjou, Canada). For each experiment, three replicates were performed for each solution at each temperature. The experiments were repeated a minimum of three times using cells from different passages. Latex beads (15 μm diameter; Beckman Coulter, Miami, Florida) were used as calibrators to convert relative

volumes to actual volumes in 1X PBS and in the experimental solutions.

Determination of the osmotic parameters

Measurements of cell volumes as a function of time were used to determine the osmotic parameters. Least squares error fits using EXCEL Solver, was used to solve for L_p and v_b, using equations 1 and 2 respectively. The analysis of the concentration dependence used 2-5X PBS solutions (583-1434 mOsm/kg) at temperatures of 4.8±0.6, 12.9±1.4, 23.3±1.2, and 37.4±0.8°C, and additional analysis with temperatures of 4.6±0.7, 8.1±0.7, 11.1±0.6, 16.4±0.5, 19.4±0.8 and 28.8±0.6°C was performed for 3X PBS solutions only. Curves were fitted for L_p and V_b for each experimental solution at each of the experimental temperatures.

The Arrhenius activation energy for L_p, described by equation 3, was fit for using linear regression of the natural logarithm of L_p as a function of the inverse absolute temperature in EXCEL.

Statistical analysis

Statistical comparisons used a standard one-way analysis of variance (ANOVA) at 5% level of significance. Estimates of L_p and v_b were compared between experimental solutions (2-5X PBS) for all the experimental temperatures.

2.3 Results

Isotonic volume

Figure 2-1 shows a representative volume distribution of TF-1 cells under isotonic conditions in 1X PBS (calibration factor=7.8; mean volume=806.1 μm^3).

The distribution was lognormal and narrow compared to other cell types, which have a more broad distribution (unpublished data). This is the result of synchronizing the cells in G_0/G_1 phases of the cell cycle. For the entire data set, the isotonic volume for TF-1 cells was 776±36 μm^3.

Hydraulic conductivity

Changes in mean cell volume as a function of time were used to calculate the L_p. Figure 2-2 is a representative graph of TF-1 cells exposed to 3X PBS at four different temperatures, which show the increase rate of cell volume shrinkage at higher temperatures, demonstrating a higher L_p. Data from experimental solutions (2-5X PBS) at four different temperatures (4.8±0.6, 12.9±1.4, 23.3±1.2, and 37.4±0.8°C) were used to examine the concentration and temperature dependence of L_p. The L_p was determined by fitting the data to Equation 1 using the least squares method with EXCEL Solver for each experimental solution and temperature and summarized in Table 2-1. Values for each concentration were pooled since there was no concentration dependence ($p>0.05$).

Osmotically-inactive fraction

For each sample, v_b was fit to Equation 2 using the least-squares method in EXCEL Solver. Data from temperatures of 4.8±0.6, 12.9±1.4, 23.3±1.2, and 37.4±0.8°C for 2-5X PBS solutions were used in order to determine the temperature and concentration dependence of v_b. The data in Table 2-2 show that the osmotically-inactive fraction was not dependent on concentration ($p>0.05$). As a result, overall data for all temperatures and concentrations were

pooled. TF-1 cells had a mean v_b of 0.35±0.03. A Boyle van't Hoff plot of equilibrium volume as a function of inverse osmolality for the aggregate data is shown in Figure 2-3. TF-1 cells responded as ideal osmometers over a range of 583-1434 mOsm/kg. The v_b could also be determined by extrapolating the slope back to the y-axis in which the intercept was 0.37. This value was within the error found using the least squares method.

Arrhenius activation energy

The L_p follows the Arrhenius equation over the range of experimental temperatures. The Arrhenius activation energy of L_p was determined using pooled data from experiments based on cell volume kinetics of 2-5X PBS solutions at temperatures 4.8±0.6, 12.9±1.4, 23.3±1.2, and 37.4±0.8°C and of 3X PBS solutions at temperatures 4.6±0.7, 8.1±0.7, 11.1±0.6, 16.4±0.5, 19.4±0.8 and 28.8±0.6°C. Figure 2-4 shows Arrhenius plots of mean L_p values for all the experimental temperatures examined. The value of E_a for L_p from these data was 13.4 kcal/mol. Figure 2-4 also demonstrates that the osmotically-inactive fraction is independent of temperature using linear regression.

2.4 Discussion

The hydraulic conductivity reported in this study was found to be strongly dependent on all temperatures reported in this study, but independent of concentration, which has been previously reported for other cells types [19]. The value for L_p was 0.342 μm/min/atm at 20°C. The L_p is within the range reported for mammalian cells, such as rat megakarycytoporietic cells, Chinese hamster lung fibroblast cells, bovine immature oocytes, chondrocytes, corneal endothelial,

epithelial and stromal cells [28]. The rate of water movement is considered slow responding, thus the Coulter counter was an efficient method of monitoring changes in cell volume. However,

TF-1 cells follow the Boyle van't Hoff relationship and thus the cells behave as ideal osmometers. The osmotically-inactive fraction can be determined using the Boyle van't Hoff plot and the least-squares method. The v_b for TF-1 cells was determined as 0.35±0.03 and 0.37, respectively. The value of v_b reported here is within the range for a variety of mammalian cell types (0.2-0.41) [4,6,8,12,19,24,28].

The Arrhenius activation energy for L_p of 13.4 kcal/mol reported here, is within normal ranges for other types of mammalian cells (12-16 kcal/mol) [4,12,19]. It has been reported that cells with an E_a of <6 kcal/mol for L_p, are fast responding, and may exhibit channel-mediated water transport [5]. Also, cells with an E_a >10 kcal/mol for L_p, such as the TF-1 cells, are slow responding and may transport water by solubility-diffusion through the plasma membrane. The high E_a for L_p indicates that the water permeability of the plasma membrane of TF-1 cells is highly dependent on temperature. However, the E_a for L_p alone may not be enough to negate the presence of aqueous pores and further analysis is required of slow responding cells to explore the possibility of other types of pores [5].

The osmotic parameters reported here for TF-1 cells are comparable with those previously reported for HSCs. The value reported here for L_p is comparable with the L_p previously reported for cord blood $CD34^+$ cells of

0.168±0.03 μm/atm/min at 20°C [14], indicating that the rate of water movement is similar for TF-1 cells and for other HSC. Based on Lp values reported by Hunt et al. at two temperatures [14], we calculated the E_a for L_p in cord blood $CD34^+$ cells to be 18.8 kcal/mol. The E_a for L_p also denotes a slow-responding cell. The v_b is comparable to that previously reported for both bone marrow hematopoietic $CD34^+$ cells of 0.205 [6] and umbilical cord blood $CD34^+$ cells of 0.32 [14] and 0.27±0.01 [14]. The average size of TF-1 cells (776±36 μm^3) was higher than that previously reported for both bone marrow $CD34^+$ cells of 345 μm^3 [6] and umbilical cord blood $CD34^+$ cells of 274±13 μm^3 [14]. This indicates that although there are differences in cell volume between TF-1 cells and $CD34^+$ cells from patient samples, the osmotic parameters for both cell types are comparable.

The parameters determined in this study are sufficient to be used in the mathematical analysis of the TF-1 cellular responses to low temperatures and ultimately in designing a cryopreservation procedure specific to these cells. The parameters summarized in Table 2-3 were subsequently used in simulations described in Chapter 3. The simulations model the cellular responses to ice formation in the extracellular solution based on how the cells responded osmotically to hypertonic solutions. It is also possible to use the osmotic parameters from other cell types to model their cellular response to low temperatures.

TABLE 2-1. L_p values for TF-1 cells (mean ± SD)

Lp	**Temperature**			
(μm/min/atm)	4.84±0.62°C	12.9±1.4°C	23.3±1.2°C	37.4±0.8°C
2X PBS	0.081±0.013	0.122±0.011	0.337±0.061	1.17±0.14
3X PBS	0.079±0.011	0.134±0.020	0.375±0.060	1.39±0.30
4X PBS	0.077±0.008	0.122±0.017	0.428±0.048	1.42±0.28
5X PBS	0.076±0.014	0.119±0.014	0.379±0.036	1.50±0.30
pooled mean	0.078±0.012	0.123±0.015	0.388±0.052	1.36±0.26

p>0.05 for all values implying no concentration dependence

TABLE 2-2. v_b values for TF-1 cells (mean ± SD)

V_b	Temperature			
	4.84±0.62°C	12.9±1.4°C	23.3±1.2°C	37.4±0.8°C
2X PBS	0.398±0.042	0.332±0.058	0.327±0.016	0.321±0.041
3X PBS	0.378±0.040	0.383±0.022	0.348±0.030	0.338±0.050
4X PBS	0.357±0.027	0.376±0.051	0.341±0.018	0.330±0.021
5X PBS	0.361±0.026	0.384±0.058	0.371±0.010	0.313±0.028
pooled mean	0.373±0.034	0.368±0.052	0.347±0.020	0.326±0.037

$p>0.05$ for all values implying no concentration dependence

TABLE 2-3. Osmotic parameters for TF-1 cells used in simulations.

Isotonic Volume	**776 μm^3**
Inactive Fraction	**0.350**
L_p (20°C)	**0.342 μm/min/atm**
Activation Energy for L_p	**13.4 kcal/mol**
Isotonic osmolality	**0.301 osm/kg**

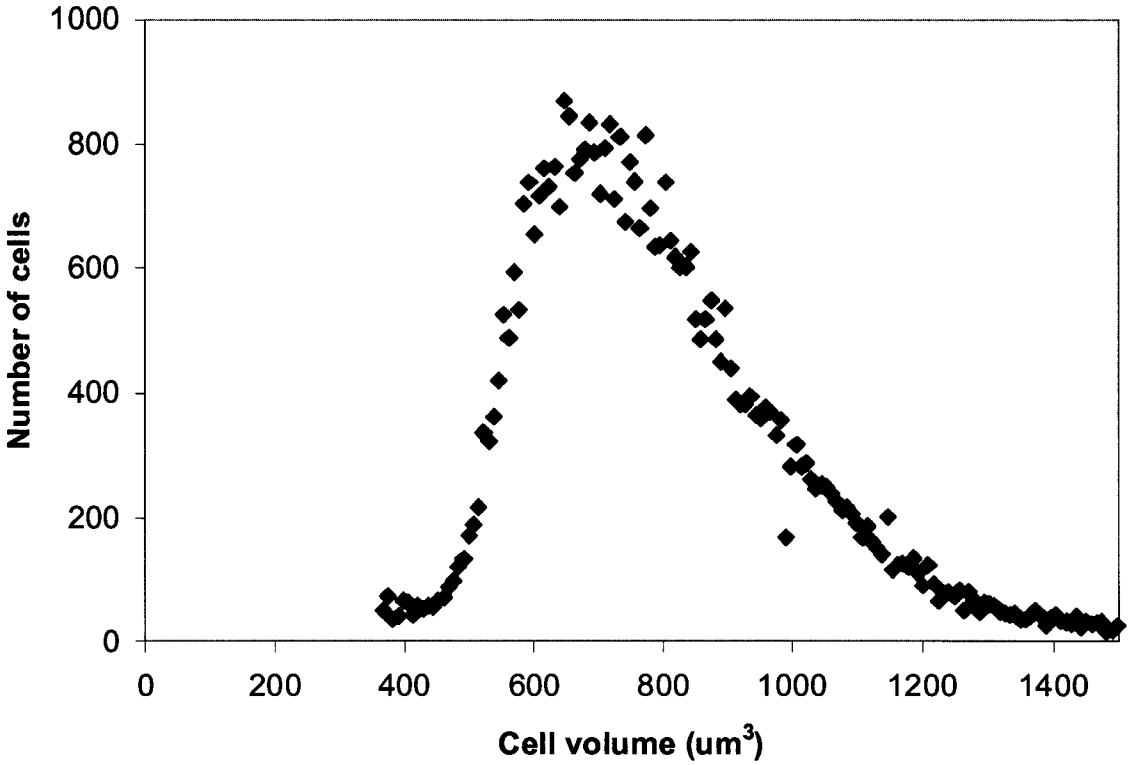

Figure 2-1. A representative graph for TF-1 cells volume distribution, with a mean of 806.1 μm^3.

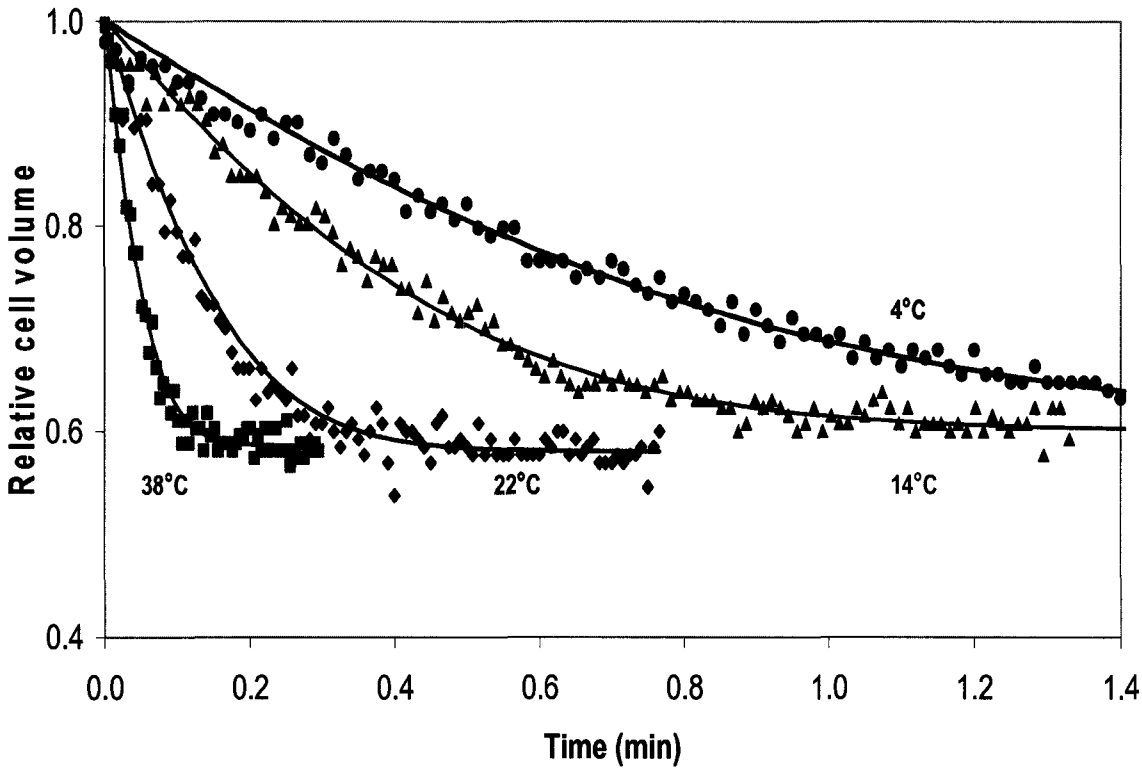

Figure 2-2. A representative graph of cell volume kinetics of TF-1 cells exposed to 3X PBS at 4 different temperatures. The symbols show the experimental volume measurements averaged over 300 msec time intervals and the solid lines represent a fitted curve to data using the least-squares error method.

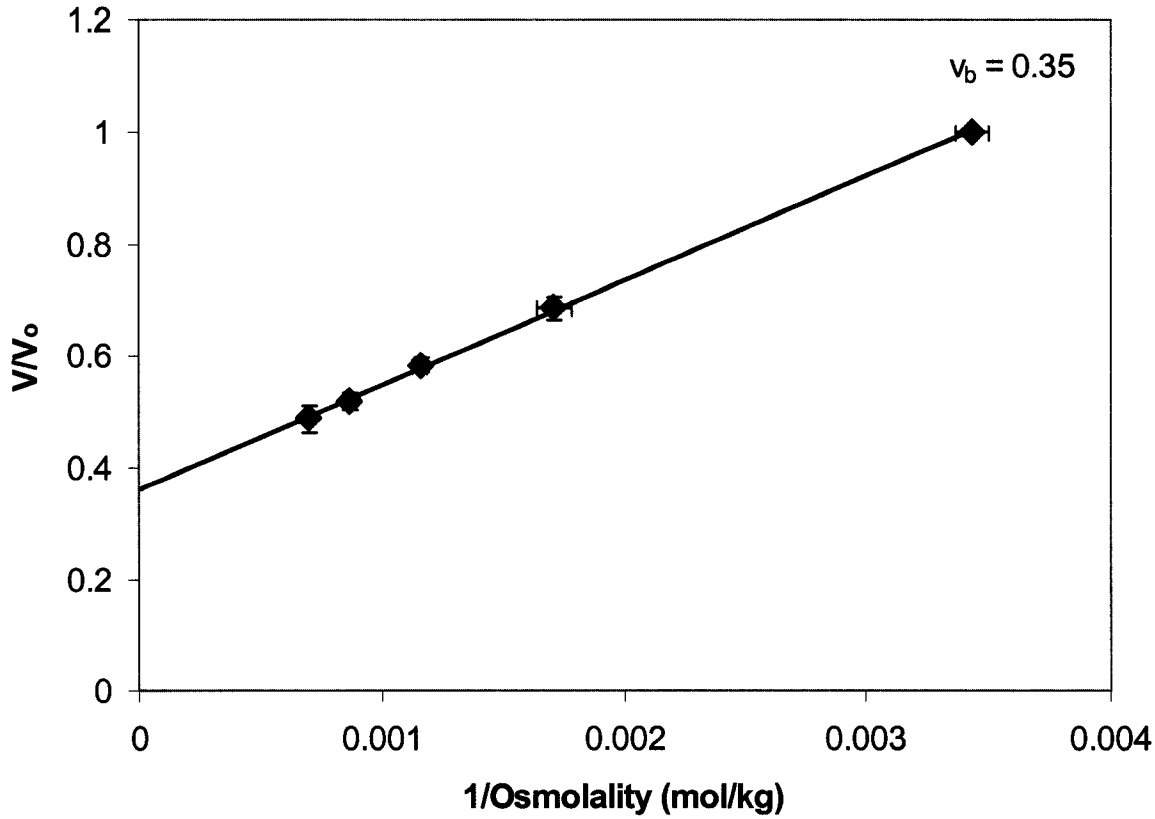

Figure 2-3. Boyle van't Hoff plot for TF-1 cells exposed to 1-5X PBS solutions. The symbols are the equilibrium volumes of the cells (±SD) and the solid line represents the linear regression fit to the equilibrium volume of the cells in hypertonic solutions. The y-intercept represents the osmotically-inactive fraction.

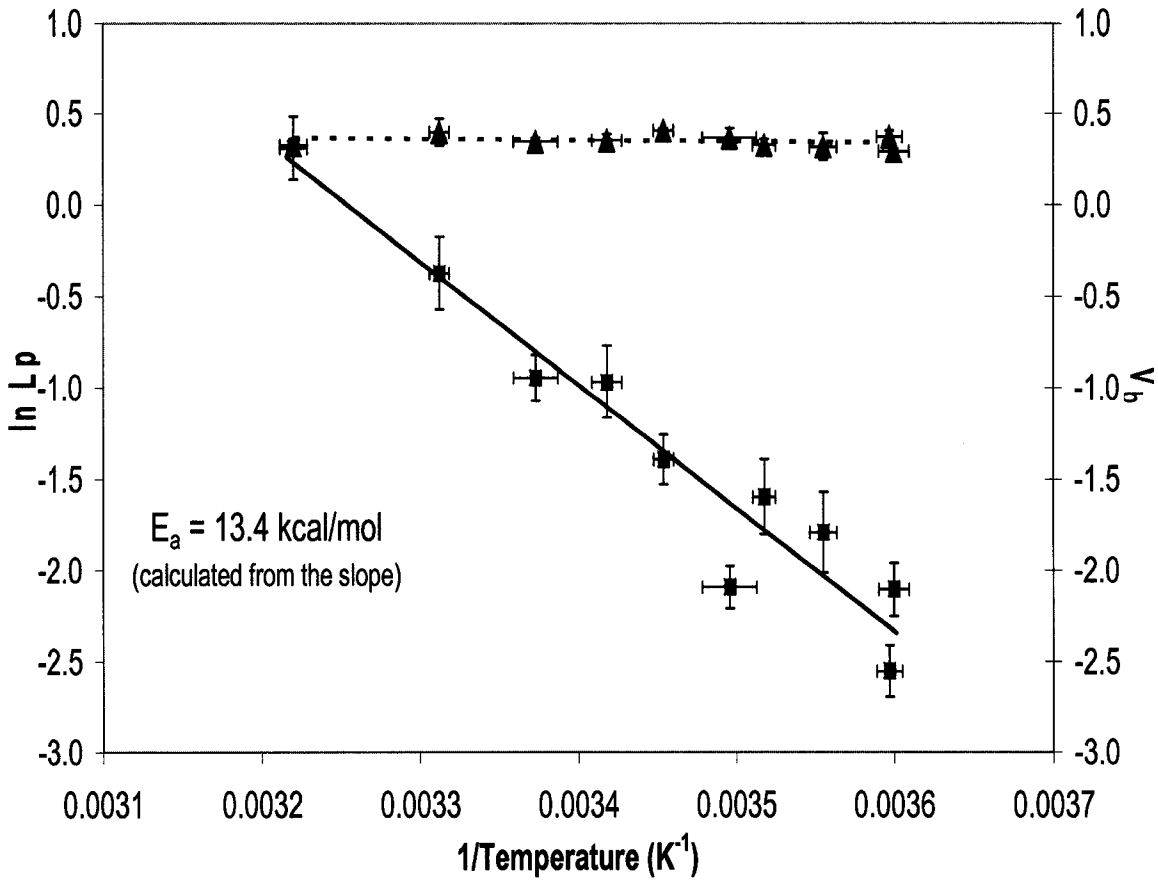

Figure 2-4. Arrhenius plot of the natural logarithm for L_p (μm/min/atm) of TF-1 cells (±SD) as a function of inverse temperature (K^{-1}) (±SD). The solid line represents the linear regression of the data for calculation of the activation energy (E_a) from the slope (R^2=0.93). Also included is the v_b as a function of inverse temperature (K^{-1}) (±SD). The dashed line represents the linear regression (R^2=0.06) of the data points, indicating no temperature dependence.

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Chapter 3: Simulations of cellular responses to low temperatures

3.1 Introduction

The freezing of cells in suspension has largely been approached empirically. However, simulations have been used to mathematically predict cellular responses to low temperatures for a variety of cell types: bovine erythrocytes [7]; yeast [15]; hamster ova [16]; hamster pancreatic islet cells [8]; and epithelial, endothelial and stroma cells [5]. Modeling is based on the theoretical response of the cell to a changing extracellular environment. The cellular responses to the formation of extracellular ice in surrounding solution are largely dependent on the movement of water across the plasma membrane. Extracellular ice formation increases the concentration of solutes in the residual liquid, resulting in osmotic efflux of water from the cell. The properties of the cell membrane, specifically the osmotic parameters, govern the rate of change of cell volume. Osmotic parameters can be used in simulations to theoretically model cellular responses to low temperatures.

Simulations reduce the time and expense involved with empirical experiments. Simulations also provide a means to analyze changes in cell volume prior to empirical experimentation. Simulations provide insight into intracellular osmolalities, concentrations and rates of water movement. These results can then be used for comparisons between cryopreservation protocols and for comparison between different cell types which may be necessary if attempting to cryopreserve a heterogeneous cell population or a tissue.

Ultimately, simulations allow for unlimited theoretical protocols to be explored by controlling cooling and warming rate, experimental temperatures, and the components of the intracellular and extracellular compartments for any cell type for which the osmotic parameters are known.

Mazur has previously used simulations to explore the effects of solutions, osmotics and temperatures on cellular systems [10]. Mazur reported the rate of water loss from the cell and the change in permeability with temperature as the parameters necessary to predict changes in volume of intracellular water with temperature. Furthermore, predictions about the probability of intracellular ice formation can be made based on the amount of intracellular water and the temperature of the cell. Subsequently, Mazur indicated that rates greater than 1°C/min may generate a supercooled cytoplasm in yeast [10,11]. Supercooling is the amount a solution can be cooled below its freezing point without ice forming and it used as an indicator of the potential for intracellular ice formation [12]. Intracellular supercooling is the extent to which a cell is cooled below the phase-change temperature before the formation of intracellular ice. Mazur also reported that there was a 10°C limit to supercooling above which the risk of intracellular ice is increased [10,11]. Cooling rates of approximately 1°C/min should reduce the amount of supercooling therefore they reduce the risk of intracellular ice. Diller further examined the probability of intracellular ice formation based on the synergistic interaction of cooling rates and supercooling [3]. During cooling, the cell attempts to maintain equilibrium across the plasma membrane either through osmotic dehydration or the formation of ice. Therefore,

with no supercooling, there is a high probability that the cell will dehydrate; whereas with greater than 10°C supercooling, the cell may form intracellular ice [3]. Ebertz also reported the use of supercooling as an indicator for intracellular ice formation for simulations on corneal endothelial, epithelial and stromal cells [5].

Based on Mazur et al.'s 'two-factor hypothesis', solution effects injury must also be considered along with intracellular ice formation injury, when attempting to determine the optimal cooling rate for cryopreserving a cell type [12]. During slow cooling, cell injury is due to prolonged exposure to high solute concentrations, as a result of cell dehydration due to extracellular ice formation. This work represents a novel approach of combining supercooling with intracellular electrolyte concentration in the presence of extracellular ice, to use as indicators of cryoinjury.

The objective of these simulations was to theoretically determine the cellular responses of TF-1 cells at various stages of the graded freezing protocol for comparison with experimental data. Simulations were performed using the osmotic parameters of TF-1 cells reported in Chapter 2 (Table 2-3). Maximum levels of intracellular electrolyte concentrations ($[KCl]_i$) and of supercooling were examined upon cooling the cells to -40°C, as indicators for solution effects injury and intracellular ice formation injury, respectively.

3.2 Calculations of low-temperature responses

Methods

The *CryoSim5* program (Dr. Locksley McGann, University of Alberta, Canada) was used to perform the simulations and Figure 3-1 is a representative image of the program interface. The program uses the phase diagrams of the components of the intracellular and extracellular solutions, the osmotic characteristics of the cell membrane and the temperature dependencies of the parameters. The simulations are calculations of the cellular osmotic responses to the concentration of solutes in the residual liquid in the presence of ice at low temperatures. Ice nucleation is assumed to be at the freezing point of the extracellular solution. Phase diagrams were used to calculate concentrations in the liquid phase for sodium chloride (NaCl)-H_2O [18] and potassium chloride (KCl)-H_2O [18] for the extracellular and intracellular compartments, respectively. The amount of intracellular protein has been reported to be more than half the dry weight of the cell [1]. It has also been reported that red blood cells possess approximately 0.0073 mol of hemoglobin per kg of intracellular water (7.3 mmolal) [2,14,17]. Since the intracellular protein content is unknown for TF-1 cells to our knowledge, we used half the molality of hemoglobin in red blood cells (3.65 mmolal) for the simulations. The hydraulic conductivity (L_p) was used to calculate osmotic cellular responses to changes in the extracellular conditions. The Arrhenius activation energy (E_a) for L_p was used to describe the temperature dependency of hydraulic conductivity. The

numerical values of the hydraulic conductivity and the E_a for L_p from Chapter 2 (Table 2-3), were extrapolated to lower subzero temperatures.

Temperature profiles

To explore the role of low and high cooling rates typically used to cryopreserve cells, we simulated the empirical procedure of the graded freezing protocols. Graded freezing provides insights into the two types of freezing injury which can affect cell recovery: solution effects and intracellular ice formation [13]. The graded freezing technique involved cooling (ie. 1 °C/min) the samples to various subzero temperatures before being either thawed directly in a 37 °C water bath or plunged into liquid nitrogen first and then thawed [13]. With this procedure it is possible to separate injury sustained during the initial cooling phase to subzero experimental temperatures, from that sustained upon further cooling to storage temperatures. Various cooling rates can also be used to explore the effect of time spent during cooling on cell recovery. Simulations were performed in which cells with no cryoprotectant were cooled to various subzero temperatures ranging from -4°C to -30°C at cooling rates ranging from 0.2°C/min to 100°C/min, prior to being plunged to -40°C at 325°C/min to model graded freezing [4].

In the experimental procedure, the samples are first placed in a -3°C methanol bath from 0°C and allowed to equilibrate prior further cooling to the various experimental subzero temperatures. The temperature profile of this equilibration is governed by Fourier's Law. Fourier's Law describes the rate of heat transfer which depends on the temperature distribution of the system [6]:

$$\frac{dT}{dt} = k \cdot \Delta T \qquad (1)$$

where dT/dt is the rate of change in temperature of the sample with time, k is the fitting constant, and ΔT is the difference in temperature between the bath and the sample. The constant was determined by monitoring the cooling profile of a sample taken from 0°C and placed in a -3°C methanol bath with a Type T thermocouple (Omega, Laval, Canada). This profile was then fitted to a curve using equation 1. The constant was then used in simulations to model the equilibration step of the graded freezing procedure. Simulating the equilibration step using Fourier's Law is a novel approach.

3.3 Results

Cooling profiles

Cells were cooled to -3°C from 0°C (k=7) and then cooled at 0.2, 0.5, 1.0, 5.0, 10, 20, or 100°C/min to plunge temperatures ranging from -4°C to -30°C, prior to being cooled rapidly to -40°C. Figure 3-2 is a representative temperature cooling profile for equilibration and cooling at 1°C/min to various subzero experimental temperatures. The cooling profiles were similar for all cooling rates except that the length of time needed to reach the intermediate temperatures decreased with increasing cooling rate.

Cell volume during cooling

Figure 3-3 is a representative graph for 1°C/min demonstrating the changes in cell volume as a function of temperature. The data showed that cells did not reach the same volume when cooled to -3°C prior to rapid cooling to

-40°C, as did cells cooled to other subzero temperatures. The cell volumes for the other temperatures were very close to the values obtained for -30°C, which was the minimal cell volume recorded. This may indicate that cells cooled to -3°C may have a greater amount of supercooling at low subzero temperatures due to the higher water content than cells cooled to the other temperatures. The results not shown for 0.2 and 0.5°C/min demonstrated similar changes in cell volume. The results not shown for 1.0, 5.0, 10, 20, or 100°C/min demonstrated less of a reduction in cell volume when cooled to the subzero temperatures.

Supercooling & $[KCl]_i$ during cooling

Supercooling was calculated in cells cooled to the plunge temperatures prior to being plunged to -40°C (325°C/min) for all cooling rates. A representative graph demonstrating supercooling as a function of temperature for cells cooled at 1°C/min is shown in Figure 3-4a. At low cooling rates, supercooling is greater than 10°C for cells initially cooled to -3°C prior to plunging. Also, at the higher cooling rates such as 100°C/min, supercooling was also seen at plunge temperatures of -6°C through -12°C, respectively (data shown for 100°C/min in Figure 3-4b).

A representative graph demonstrating $[KCl]_i$ as a function of temperature for cells cooled at 1°C/min is shown in Figure 3-5a. Cells cooled to increasingly lower subzero plunge temperatures showed increasing concentrations of $[KCl]_i$, with the highest concentration for cells cooled to -30°C at 1°C/min. This correlates with the decrease in cell volume reported in the previous section. This gradual increase in $[KCl]_i$ demonstrates the potential for increased solution

effects upon cooling to the lower subzero temperatures. Results for 0.2°C/min and 0.5°C/min were not shown however were similar to 1°C/min data, but over a longer period of time. Figure 3-5b shows the $[KCl]_i$ as a function of temperature for cells cooled at 100°C/min. This demonstrates the increase of $[KCl]_i$ is minimized with higher cooling rates.

Maximum supercooling and $[KCl]_i$ during cooling

The maximum amount of supercooling was calculated as the highest amount of supercooling which occurred throughout the cooling profile for each plunge temperature. Figure 3-4a and 3-4b shows supercooling as a function of temperature for TF-1 cells cooled at 1.0°C/min and at 100°C/min respectively, with arrows indicating where the maximum supercooling was determined for the various plunge temperatures. The maximum supercooling for each cooling rate was then summarized and graphed as a function of plunge temperature (Figure 3-6). Similar patterns of high supercooling (27°C) at -3°C were demonstrated for all the cooling rates, with a decrease to below 10°C at approximately -4°C for cells cooled at 0.2, 0.5, and 1.0°C/min and at approximately -13°C for cells cooled at 100°C/min. Intracellular ice formation thus only appears to play a role in freezing injury for cells cooled to high subzero temperatures for low cooling rates and intermediate subzero temperatures for higher cooling rates.

The maximum amount of $[KCl]_i$ was calculated as the highest concentration of KCl which occurred throughout the cooling profile for each plunge temperature. Figure 3-5a and 3-5b shows the $[KCl]_i$ as a function of temperature for TF-1 cells cooled at 1.0°C/min and at 100°C/min respectively,

with arrows indicating where the maximum $[KCl]_i$ was determined for the various plunge temperatures. The maximum $[KCl]_i$ for each cooling rate was then summarized and graphed as a function of plunge temperature (Figure 3-7). Similar patterns of an increase in $[KCl]_i$ are demonstrated for all cooling rates, which is consistent with the change in cell volume. Cooling rates of 0.2, 0.5, and 1.0°C/min showed the sharpest increase in $[KCl]_i$ at the higher subzero temperatures, compared with higher cooling rates that showed a gradual increase, which is consistent with the cell dehydrating sufficient to maintain equilibrium. Thus at the low cooling rates examined, cells were exposed to comparably high solute conditions at high subzero temperatures. However, there was increased time spent exposed to the solutes for the 0.2°C/min compared with the 1.0°C/min, so one would expect that 0.2°C/min would have a lower cell recovery because cells would have been exposed to increasingly high solute concentrations for a longer period of time. The optimal temperature for plunging the cells after the initial cooling phase is a function of temperature and the amount of time spent cooling to that temperature, which influences $[KCl]_i$ and supercooling.

3.4 Discussion

Based on the simulations with multiple cooling rates, the amount of supercooling indicates that intracellular ice formation won't play role in the freezing injury at temperatures below -13°C. There is evidence of extensive supercooling for cooling rates of 0.2, 0.5, and 1°C/min, for temperatures between -3°C and -4°C. This range of temperatures is broadened for the higher cooling

rates extending to -13°C for 100°C/min. Hence, intracellular ice formation would have maximum potential for cryoinjury at high subzero temperatures. However, the levels of $[KCl]_i$ also change with cooling rate and experimental temperature and provides insight into the likelihood of solution effects. Lovelock proposed that high levels of salt (>3 M) due to ice formation may cause significant damage to red cell membranes [9]. Based on $[KCl]_i$, we would propose that cooling cells below -10°C before plunging to colder temperatures may cause significant damage to the cell membrane due to exposure to greater than 3M NaCl. We also propose that freezing cells using rates between 0.2°C/min and 1°C/min may show a significant decrease in cell survival compared with the higher cooling rates due to the sharp increase in $[KCl]_i$ at high subzero temperatures. TF-1 cells subjected to these temperatures would be exposed to increased solute concentrations and to increased exposure time based on the lower cooling rates. Since the levels of $[KCl]_i$ were comparable for the 3 low cooling rates, empirical assessment would be required to further differentiate between these low cooling rates. The variables of solute concentration, temperature and time require further investigation empirically in order to draw conclusions regarding the use of $[KCl]_i$, as an novel indicator for cryoinjury, and the potential to model the use of supercooling coupled with $[KCl]_i$ to minimize cryoinjury in freezing protocols.

Supercooling and $[KCl]_i$ can be used as indicators of potential injury sustained during freezing by intracellular ice formation and solution effects, respectively. At low cooling rates, cells are primarily exposed to high concentrations of solutes and the potential for intracellular ice formation is low

due to minimal supercooling. It is necessary to validate these results empirically to determine whether solution effects injury can be minimized by decreasing the time spent cooling (ie. 1°C/min cooling rate). We predict that the solution effects injury can be best avoided by increasing the cooling rate in order to reduce exposure to harmful concentrated solutes but still minimize supercooling.

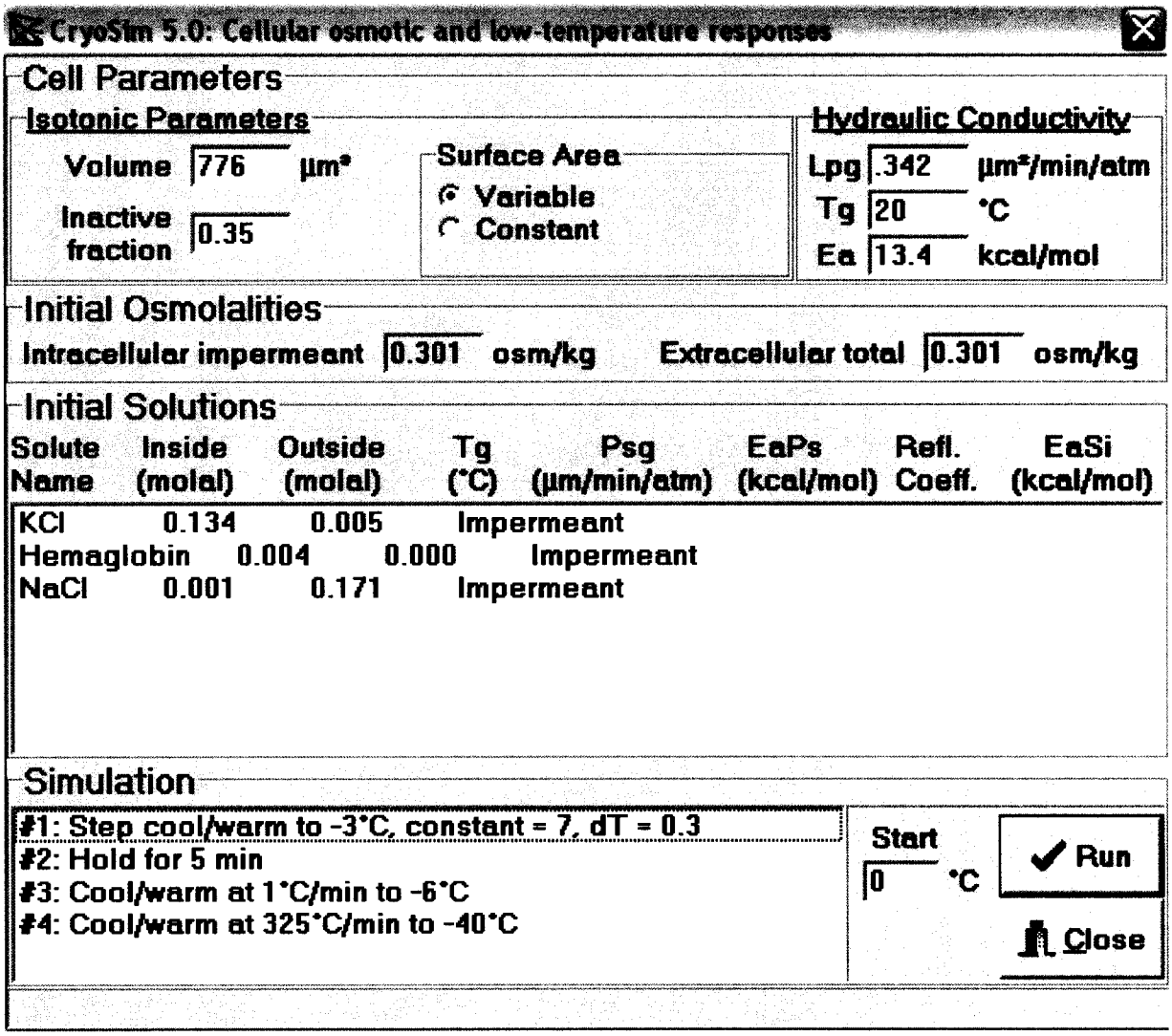

Figure 3-1. Schematic of CryoSim5 program used to simulate cellular responses.

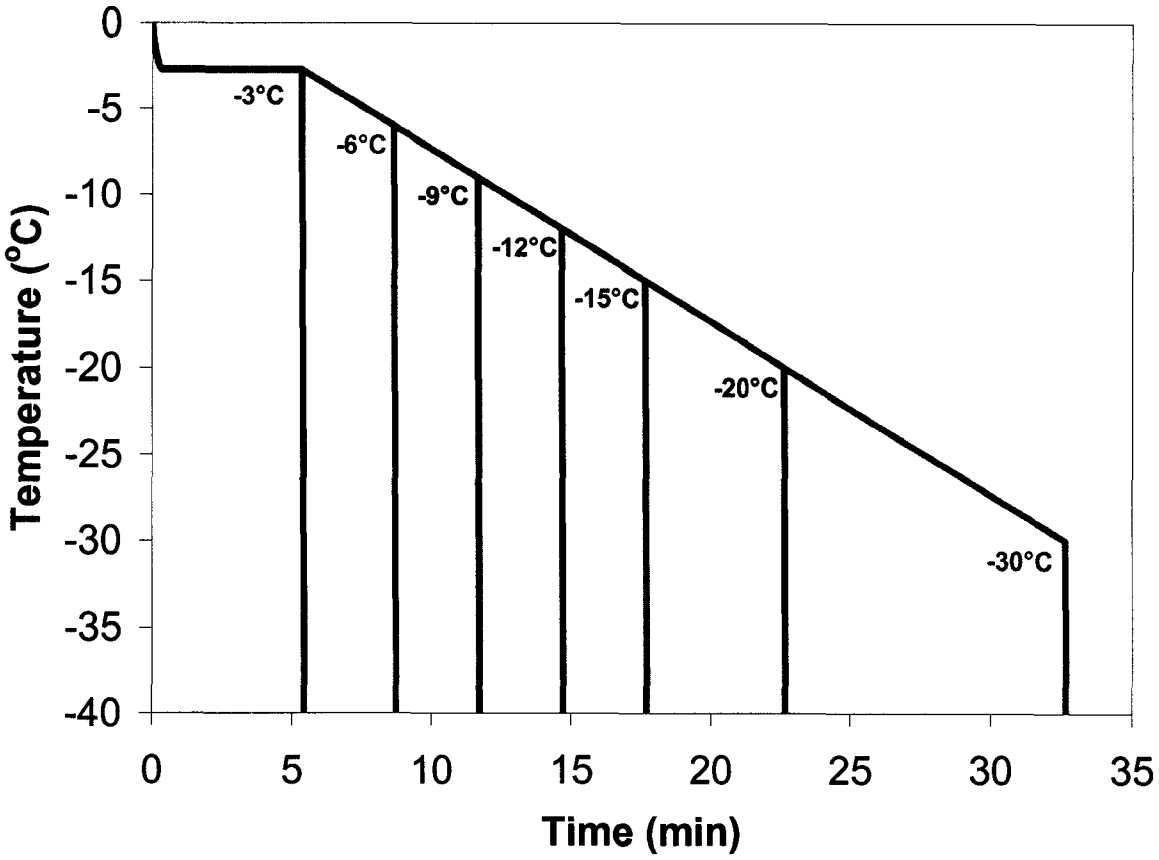

Figure 3-2. A representative graph of the cooling profile for TF-1 cells cooled using Fourier's Law (k=7) to -3°C, held for 5 minutes, cooled at 1°C/min to various subzero plunge temperatures (-4°C to -30°C)and then plunged to -40°C (325°C/min).

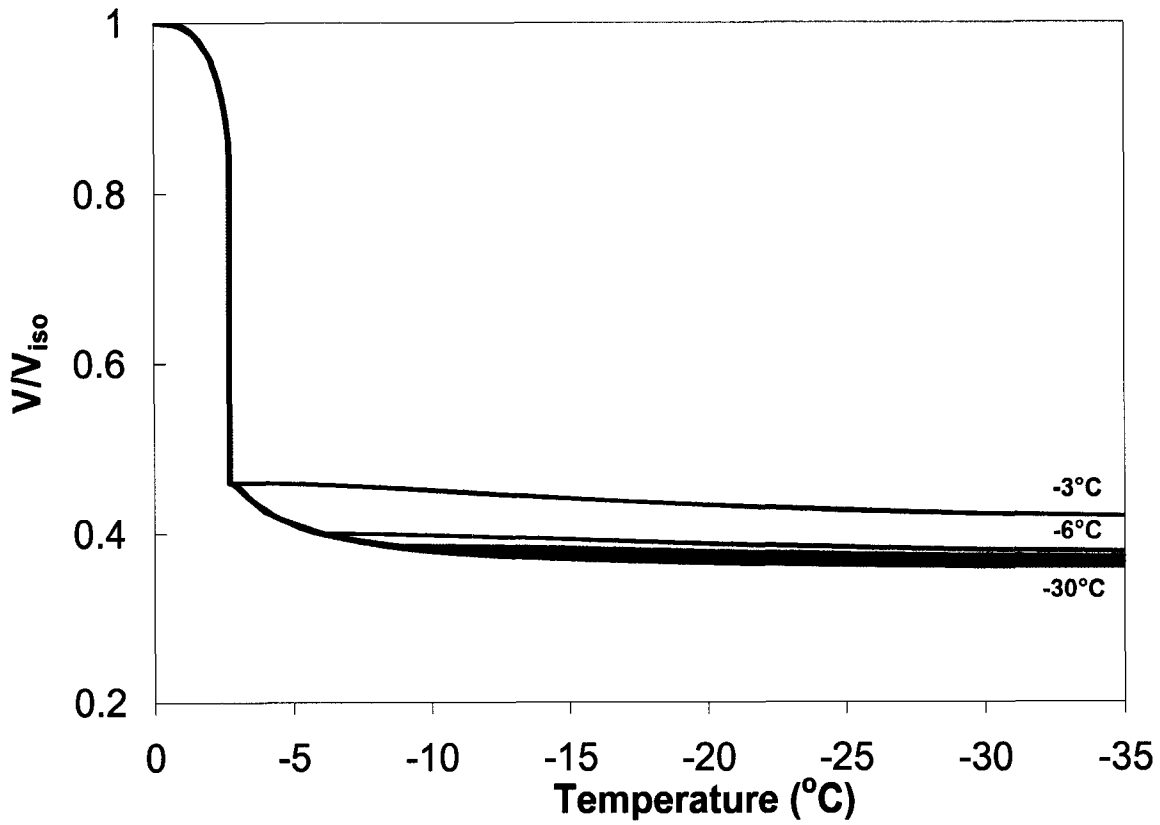

Figure 3-3. A representative graph of volume as a function of temperature for TF-1 cells being cooled using Fourier's Law (k=7) to -3°C and then cooled at 1°C/min to various subzero plunge temperatures (-4°C to -30°C), prior to being plunged to -40°C (325°C/min).

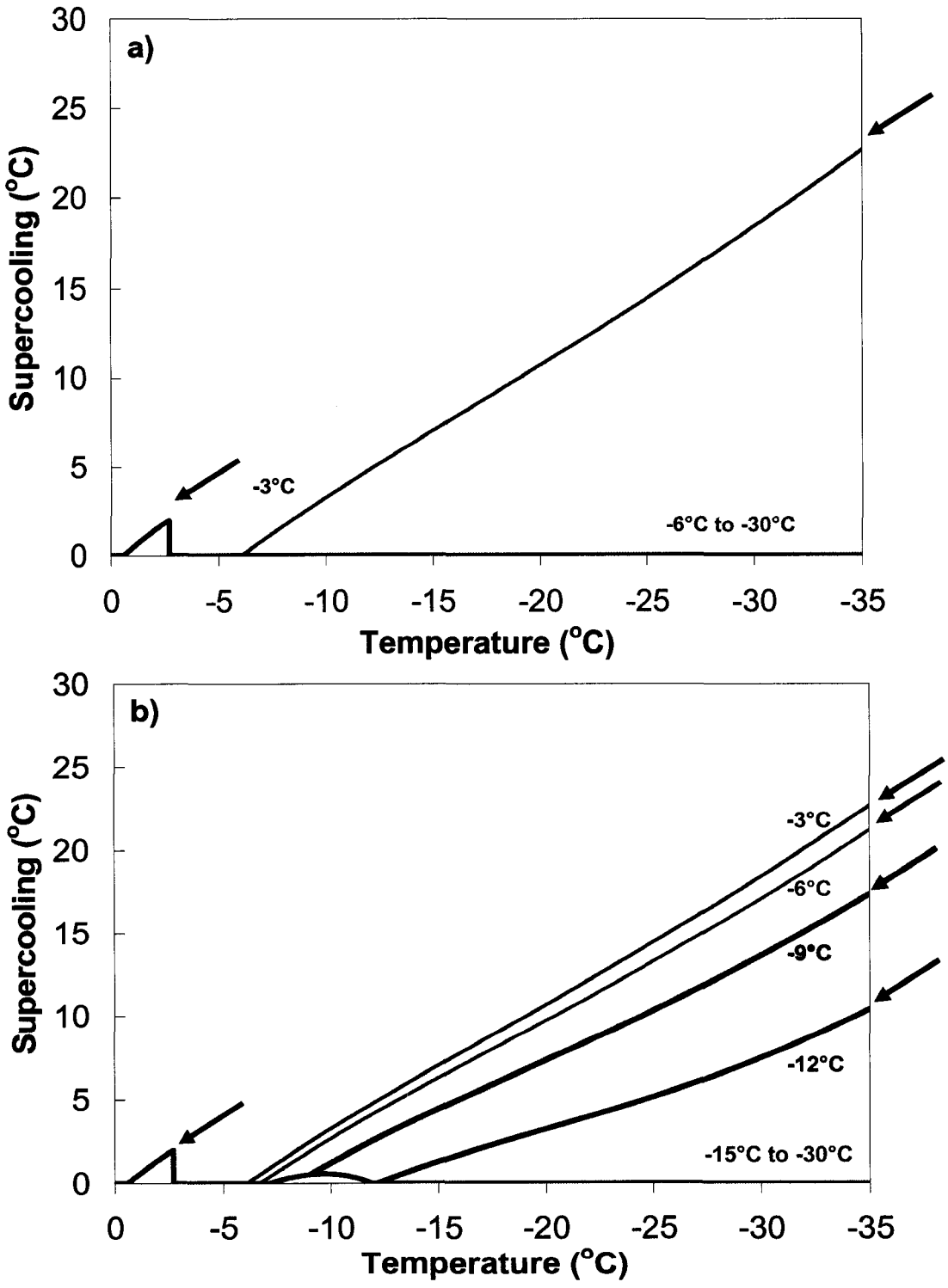

Figure 3-4. A representative graph of supercooling versus temperature for TF-1 cells cooled using Fourier's Law (k=7) to -3°C and then cooled **(a)** at 1°C/min and **(b)** at 100°C/min to various subzero plunge temperatures (-4 to -30 °C), prior to being plunged -40°C (325°C/min). Arrows indicate maximum supercooling.

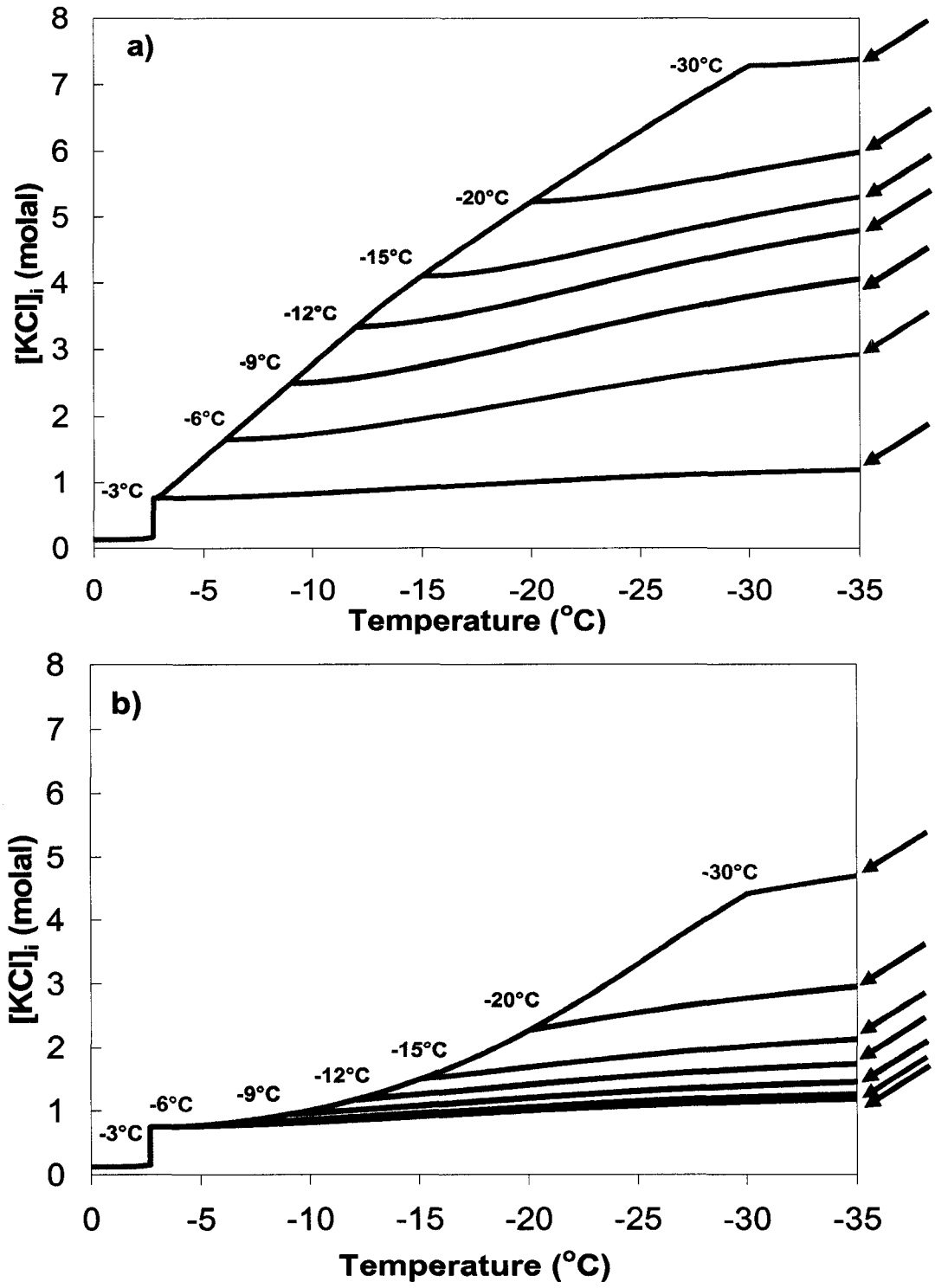

Figure 3-5. A representative graph of $[KCl]_i$ versus temperature for TF-1 cells cooled using Fourier's Law (k=7) to -3°C and then cooled **(a)** at 1°C/min and **(b)** at 100°C/min to various subzero plunge temperatures (-4°C to -30°C), prior to being plunged to -40°C (325°C/min). Arrows indicating maximum $[KCl]_i$.

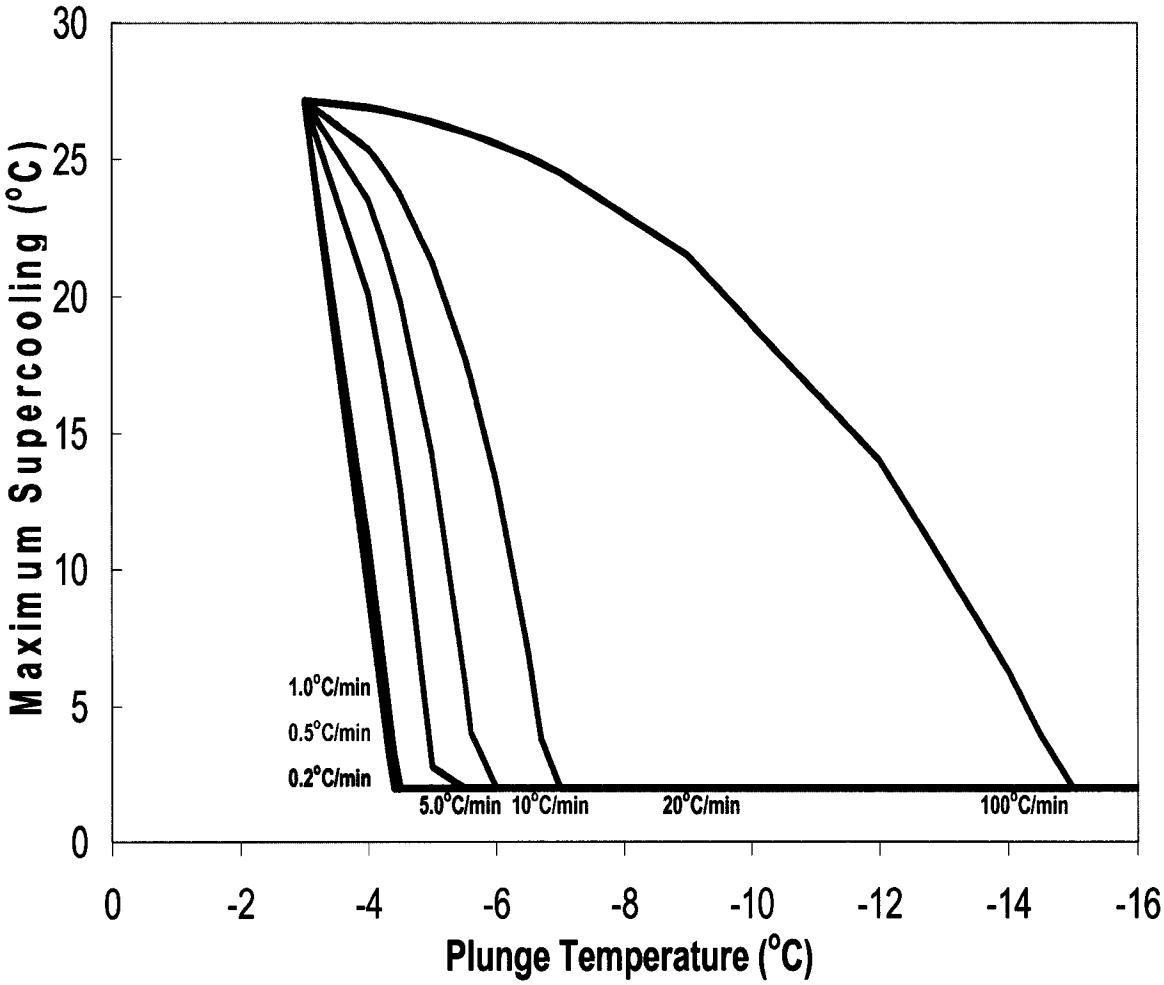

Figure 3-6. A representative graph of the maximum supercooling as a function of temperature for TF-1 cells cooled using various cooling rates to various plunge subzero temperatures (-4°C to -30°C), prior to being to lower temperatures (325°C/min). Maximum supercooling was calculated between the freezing point of the extracellular solution and -40°C.

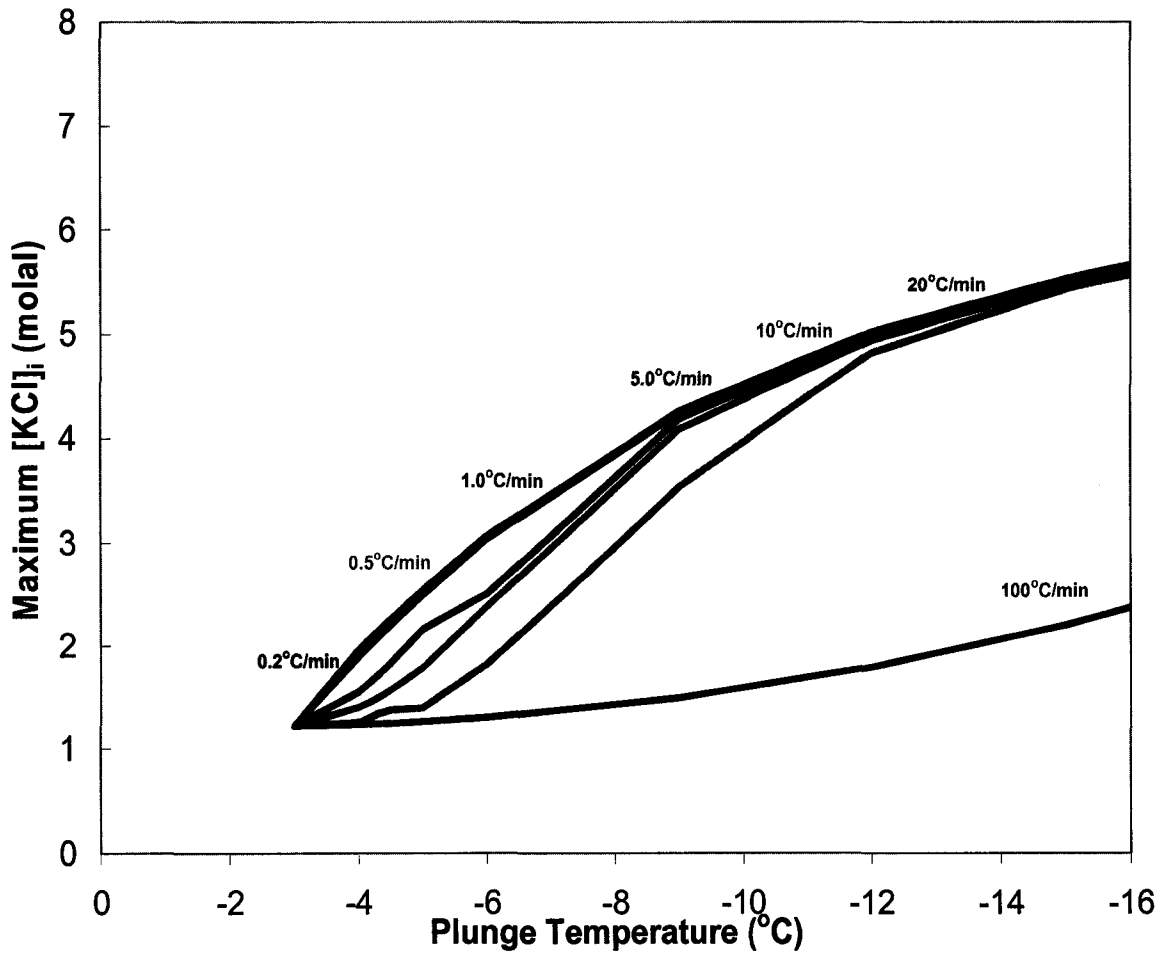

Figure 3-7. A representative graph of the maximum $[KCl]_i$ as a function of temperature for TF-1 cells cooled using various cooling rates to various subzero plunge temperatures (-4°C to -30°C), prior to be plunged to -40°C (325°C/min).

3.4 References

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Chapter 4: Experimental assessments of simulation outcomes

4.1 Introduction

Traditionally, cryopreservation protocols are determined empirically; however, interest in using simulations as a tool to approach cryopreservation is increasing. Based on mechanisms of injury that may occur during freezing, there are 4 major steps in the cryopreservation process: 1) selection and addition of a cryoprotectant; 2) selection of a cooling rate and determination of the storage temperature; 3) selection of a warming rate; and 4) removal of the cryoprotectant [9]. The cryoprotectant is an important factor in determining an optimal cryopreservation procedure as different cell types respond differently to freezing depending on the solutes present in the extracellular and intracellular environment.

Dimethyl sulfoxide (DMSO) is a penetrating cryoprotectant and is currently the standard cryoprotectant used to cryopreserve hematopoietic stem cells (HSC) for clinical use. For HSCs, it has been shown that using a 10% DMSO solution and cooling at a rate of 1°C/min provides sufficient viability for transplantation, albeit suboptimal [11,21]. However, there is significant interest in designing a cryopreservation protocol for HSCs which does not rely on DMSO as a cryoprotectant and yet yields an optimal cell recovery. It has been shown that DMSO may have adverse effects for patients undergoing high-dose chemotherapy prior to a stem cell transplant [4,8,22,26]. Also, for cord blood

(CB) samples, DMSO is washed post-thaw resulting in cell loss due to clumping. A few studies have attempted to reduce the amount of DMSO alone [1,3] or combine it with a non-penetrating cryoprotectant, such as hydroxyethyl starch (HES) [5,10,14].

In cryopreservation procedures, cells are cooled at a finite rate which is optimized for the cell type and cryoprotectant. This optimization is typically performed empirically using a variety of different cryoprotectants, at varying concentrations, and a range of cooling rates. McGann developed the graded freezing technique, which sought to explore the two types of freezing injury by determining the subzero temperature range where damage occurs [20]. The samples were slow cooled (ie. 1°C/min) to various subzero plunge temperatures before being either thawed directly or plunged into liquid nitrogen first and later thawed. This technique was later used to explore the differing actions of penetrating and non-penetrating cryoprotectants [19]. McGann reported that when freezing Chinese hamster fibroblasts with 20% HES, he was able to achieve comparable cell recovery to when using 10% DMSO/media [19].

In order for simulations to be used in cryopreservation, it is necessary to test the predictions of simulations empirically. The objective of this study was to conduct graded freezing experiments with TF-1 cells and compare the cell survival outcomes with the theoretical predictions put forward in Chapter 3 based on degrees of supercooling and intracellular electrolyte concentration ($[KCl]_i$). Membrane integrity was used as an assay for freeze-thaw injury. Membrane integrity has been used as an indicator of cell damage during freezing, as it has

been shown that the membrane is a site of freezing-thawing injury [2]. Also, it has been shown that there is a correlation between intracellular freezing and membrane damage for cells in suspension [2,18].

4.2 Materials & Methods

TF-1 cell culture

TF-1 cells (ATCC, Manassas, Virginia) were grown at 37°C in 5% CO_2 in RPMI 1640 Medium Modified (ATCC) with 10% fetal bovine serum (FBS) (ATCC), and supplemented with 2 ng/mL recombinant human GM-CSF (Stemcell Technologies, Vancouver, Canada). Cells were maintained between 0.1 x 10^6 and 1 x 10^6 cells/mL, according to ATCC guidelines. Prior to experiments, cells were washed twice with serum-free RPMI media and incubated overnight to synchronize the cells [15]. Cells were then centrifuged and re-suspended at a concentration of 4 x 10^6/mL, which was necessary for the viability assessment program to be used.

Experimental solutions

TF-1 cells were re-suspended in serum-free RPMI prior to the graded freezing experiments. In order to compare the results with the clinical standard, TF-1 cells were also re-suspended in 10% DMSO/RPMI at 4°C, prior to freezing experiments.

Graded freezing experiments

Samples of 0.2 mL cell suspension, in serum-free RPMI or 10% DMSO/RPMI, in glass tubes (Fisher, Edmonton, Canada) were cooled in a 0°C ice bath for 5 minutes. Control samples were removed and either warmed in a

37°C water bath or plunged into liquid nitrogen (325°C/min; [6]). Experimental samples were transferred into a methanol bath preset at -3°C and allowed to equilibrate for 5 minutes prior to ice nucleation with cold forceps. After 5 minutes, the bath cooled at 0.2, 0.5, or 0.9°C/min to -40°C. The cooling rates were monitored using a Type T thermocouple (Omega, Laval, Canada). Samples were then removed at -3, -6, -9, -12, -15, -20, -30, and -40°C and either thawed directly in a 37°C water bath or plunged into liquid nitrogen. Samples were kept in liquid nitrogen for a minimum of 1 hour prior to being thawed in a 37°C water bath. Duplicate samples were used for both the direct thaw and the plunge conditions at each experimental temperature. Each experiment was repeated in triplicate for each cooling rate and experimental solution. Samples in 10%DMSO/RPMI were only cooled using 0.9°C/min.

Viability assessment

Cell viability was assessed by a membrane integrity assay. The assay was performed by incubating cells with SYTO® 13 (Molecular Probes, Eugene, Oregon) and ethidium bromide (EB) (Sigma, Mississauga, Canada) [25]. Syto 13 permeates the cell membrane of all cells and complexes with DNA and fluoresces green under UV exposure. EB penetrates cells with a damaged plasma membrane and also complexes with DNA fluorescing red under UV conditions. The dual stain allows for differentiation between cells with and without intact plasma membranes.

The Syto/EB stain was prepared using 40 μL of 2.5 mM EB stock solution and 10 μL of 5 mM SYTO® 13 stock solution mixed with 350 μL phosphate-

buffered saline (PBS). Final concentrations were 0.25 mM EB and 0.125 mM Syto. Twenty μL of stain was added to 200 μL each sample, mixed, and allowed to incubate for 2 minutes at room temperature. Fluorescent images were captured using a Leitz Dialux 22 fluorescence (440-480 nm) microscope (Leitz, Germany) fitted with a PIXERA Viewfinder Pro digital camera (Pixera Corporation, Los Gatos, CA, USA) digital camera. The Viability Assessment Program (The Great Canadian Computer Company, Spruce Grove, Canada), which counts red versus green pixels was used to quantify cell membrane integrity from digital images [13]. This method measures membrane integrity of the cell remaining after experimental treatment.

4.3 Results

Conventional cryopreservation protocol with DMSO

The standard for cryopreserving HSCs is to cool the cells at 1°C/min in 10% DMSO. TF-1 cells were cooled at 0.9°C/min in 10% DMSO/RPMI to various temperatures down to -40°C, prior to being thawed directly or plunged into liquid nitrogen (Figure 4-1). The maximum percentage of membrane integrity was 63.7±9.8%, when samples were cooled to -12°C to -15°C, prior to being plunged into liquid nitrogen. The results were comparable with that previously reported for cryopreserving HSCs with 10% DMSO cooling at 1.0°C/min (79±5% [12]; 67.4±2.0% [24]. This experiment was limited by the cooling capacity of the methanol bath.

Graded freezing with no cryoprotectant using various cooling rates

TF-1 cells were suspended in serum-free RPMI and cooled at 0.2°C/min to various temperatures up to -20°C, prior to being thawed directly or plunged into liquid nitrogen. Cells thawed directly from the subzero plunge temperatures showed a 50% decrease in membrane integrity by -12°C, indicating that a major portion of cells were damaged prior to being plunged into liquid nitrogen (Figure 4-2). However, damage at higher subzero temperatures occurred as a result of the plunge into liquid nitrogen. There was limited cell recovery when the cells were plunged into liquid nitrogen at all plunge temperatures. The maximum recovery of 24.2±5.5% was seen for TF-1 cells plunged at -3°C.

Figure 4-3 shows the membrane integrity as a function of plunge temperature for TF-1 cells cooled at 0.5°C/min to -40°C, prior to being plunged into liquid nitrogen. TF-1 cells demonstrated similar membrane integrity for both thaw and plunge samples as with 0.2°C/min. The maximum recovery of 28.2±5.8% was obtained between -3°C and -9°C.

Membrane integrity as a function of plunge temperature for TF-1 cells cooled at 0.9°C/min to -40°C, prior to being thawed directly or plunged into liquid nitrogen is shown in Figure 4-4. Results were also similar to those for 0.2°C/min and 0.5°C/min. TF-1 cells showed maximum recovery of 27.8±0.8% at -9°C. This experiment was limited by the cooling capacity of the methanol bath, which had a maximum cooling rate of 0.9°C/min.

Data from all three cooling rates demonstrated a 50% decline in membrane integrity for cells thawed directly from the plunge temperature at -12°C. This

indicates that cells were damaged prior to being plunged into liquid nitrogen, possibly due to solution effects. However, there was a significant difference between the membrane integrity for cells directly thawed and those further plunged into liquid nitrogen. Due to the high cooling rate upon plunging into liquid nitrogen (325°C/min), this indicates that intracellular ice formation may play a role in damage at these temperatures. There does appear to be a zone of subzero plunge temperatures (-3°C to -9°C), which confers some protection against injury during the plunge into liquid nitrogen for all the cooling rates. This would constitute an optimal subzero plunge temperature range for these cooling rates.

4.4 Discussion

Discussion of experimental data

The conventional cryopreservation protocol of using 10%DMSO solution yielded comparable results with other HSCs cryopreservation reports. The experimental results for cryopreserving TF-1 cells without cryoprotectants were significantly lower than the standard. The data showed that the membrane integrity between the various cooling rates was within standard error mean of each other. TF-1 cells responded similarly to exposure to the various subzero plunge temperatures and to subsequent plunging into liquid nitrogen.

Comparison of theoretical and experimental results

Simulations from Chapter 3 predicted that there was no difference in maximum $[KCl]_i$ between the cooling rates, however the time spent exposed to these elevated concentrations may cause additional cryoinjury. The

experimental results demonstrated that there was not a significant difference in membrane integrity between the cooling rates of 0.2, 0.5, and 0.9°C/min, which is consistent with the theoretical results based solely on $[KCl]_i$. Simulations did indicate that there was potential for increased exposure time to the solutes with the lower cooling rate (0.2°C/min), however the experimental data demonstrated that the percentages of membrane integrity were within error for all the cooling rates. Therefore, the increased exposure time for the lower cooling rates was not significant.

For all cooling rates, simulations predicted a progressive increase in $[KCl]_i$ upon cooling to lower temperatures. Based on Lovelock's work, we predicted that salt concentrations of greater than 3 M would be damaging to the cells [17] and the experimental data demonstrated that there was a decrease in membrane integrity (~60%) at this concentration for all the cooling rates. The experimental data also demonstrated a progressive decline in membrane integrity for cells thawed directly from subzero plunge temperatures. At low subzero plunge temperatures (<-20°C), cells directly thawed had low percentages of membrane integrity (<20%). Therefore, the exposure time coupled with the concentration of solutes may have been significant variables for freezing injury.

Simulations also predicted that cells cooled to -3°C prior to being cooled at 325°C/min to -40°C would have a high degree of supercooling (27°C). The experimental results demonstrated that upon cooling to -3°C prior to plunging into liquid nitrogen, TF-1 cells had a relatively high percentage of membrane integrity. This indicates that although intracellular ice formation may have played a role in

membrane damage at this plunge temperature, there was another source of damage upon cooling to lower temperatures, where solutions effects were present. Also, the range of plunge temperatures between -3°C and -9°C, which demonstrated the highest viability, had high variations in maximum supercooling (2°C to 27°C) and in maximum $[KCl]_i$ (2 to 4 M). This alludes to the complex interactions between these two types of injury at subzero temperatures.

Conclusions

We hypothesized that by manipulating the cooling rate, DMSO could be eliminated from the protocol. Overall, the survival was still low compared to the standard cryoprotectant solution of 10% DMSO, which conferred significant protection to -40°C. Since DMSO permeates the cell, it reduces the temperature that ice is formed both in the intracellular and extracellular compartments [19]. Our results indicate that although serum-free media may promote some cell dehydration to minimize intracellular freezing, it may not offer enough protection against ice formation and/or protect against solution effects incurred during the initial cooling stage. Further simulations are required to explore the effects of subzero plunge temperatures and hold times in order to minimize injury.

Establishing cryopreservation procedures has largely been done empirically, however some studies have attempted to base their approach on the theoretical understanding of a cells low temperature responses to freezing [7,16,23]. Simulations provide a precise starting point when determining a cryopreservation protocol. Supercooling and $[KCl]_i$ are good indicators of potential damage due to intracellular ice formation and solution effects,

respectively. Furthermore, this study demonstrates the usefulness of simulations, combined with graded freezing in attempting to determine the optimal cryopreservation protocol for a particular cell type. Specifically, graded freezing provides useful insight into the mechanisms of damage conferred by slow cooling over a range of subzero temperatures [20]. It allows for the manipulation of different variables of the cryopreservation protocol, cooling rates, plunge temperatures, cryoprotectants, and storage temperatures, so that a comparison between them can be made. Ultimately, both techniques allow for an optimum protocol to be determined.

The use of DMSO as a cryoprotectant has been seen as a problem for HSC transplant patients due to its toxicity [4,8,22,26]. The survival of cryopreserved cells is largely dependent on the rate at which cells are cooled, coupled with the cryoprotective solution used. This study demonstrates that by manipulating the cooling rates when freezing TF-1 cells, membrane integrity with serum-free media is present albeit at lower values than that reported with DMSO. Therefore, the possibility of cryopreserving HSCs without DMSO requires further analysis of subzero temperatures and hold times.

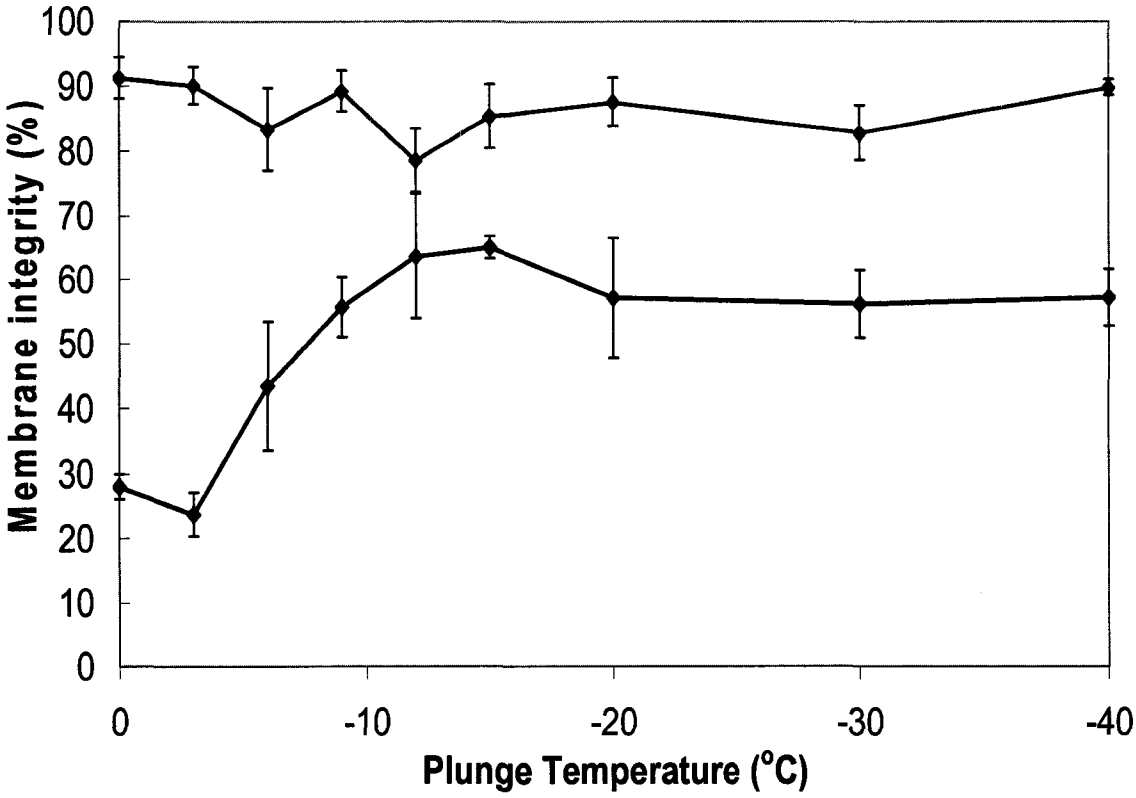

Figure 4-1. Membrane integrity of TF-1 cells (±SEM) in 10% DMSO/RPMI media after being cooled at 0.9°C/min to various subzero plunge temperatures and either thawed directly (upper curve) or plunge into liquid Nitrogen (lower curve) prior to thawing.

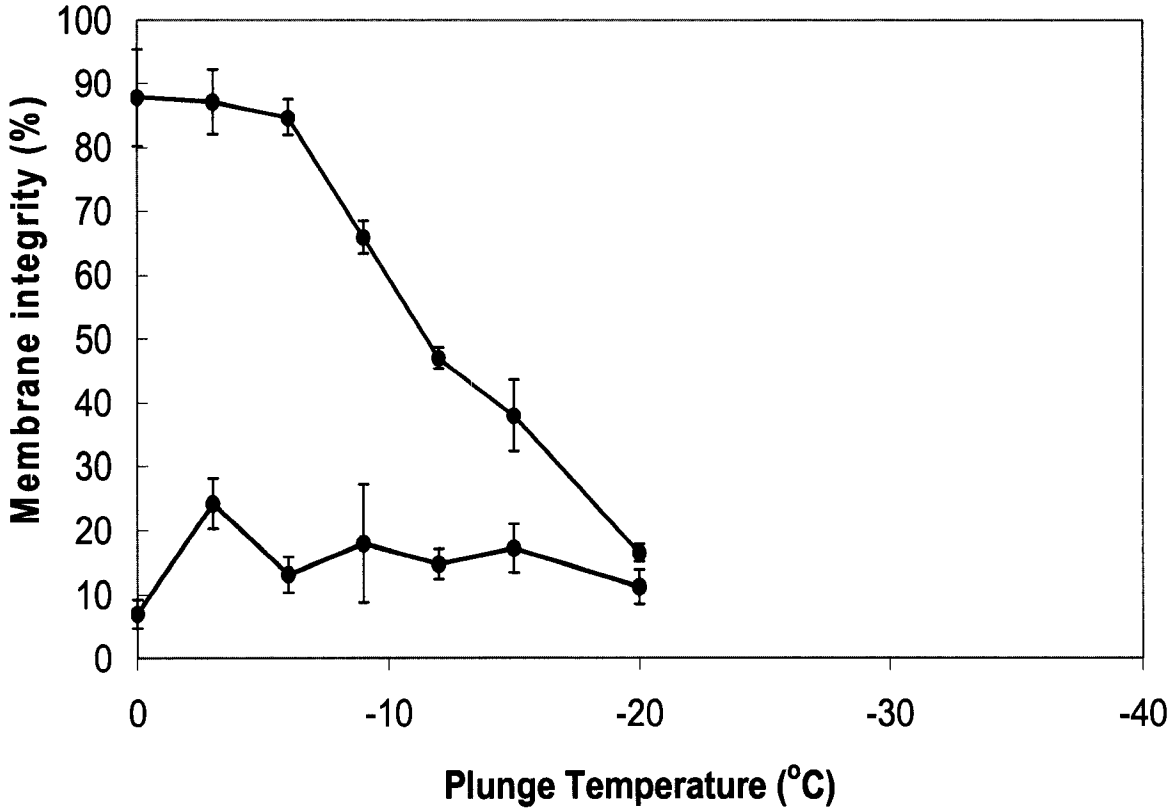

Figure 4-2. Membrane integrity of TF-1 cells (±SEM) in serum-free RPMI media after being cooled at 0.2°C/min to various subzero plunge temperatures and then either thawed directly (upper curve) or plunge into liquid Nitrogen (lower curve) prior to thawing.

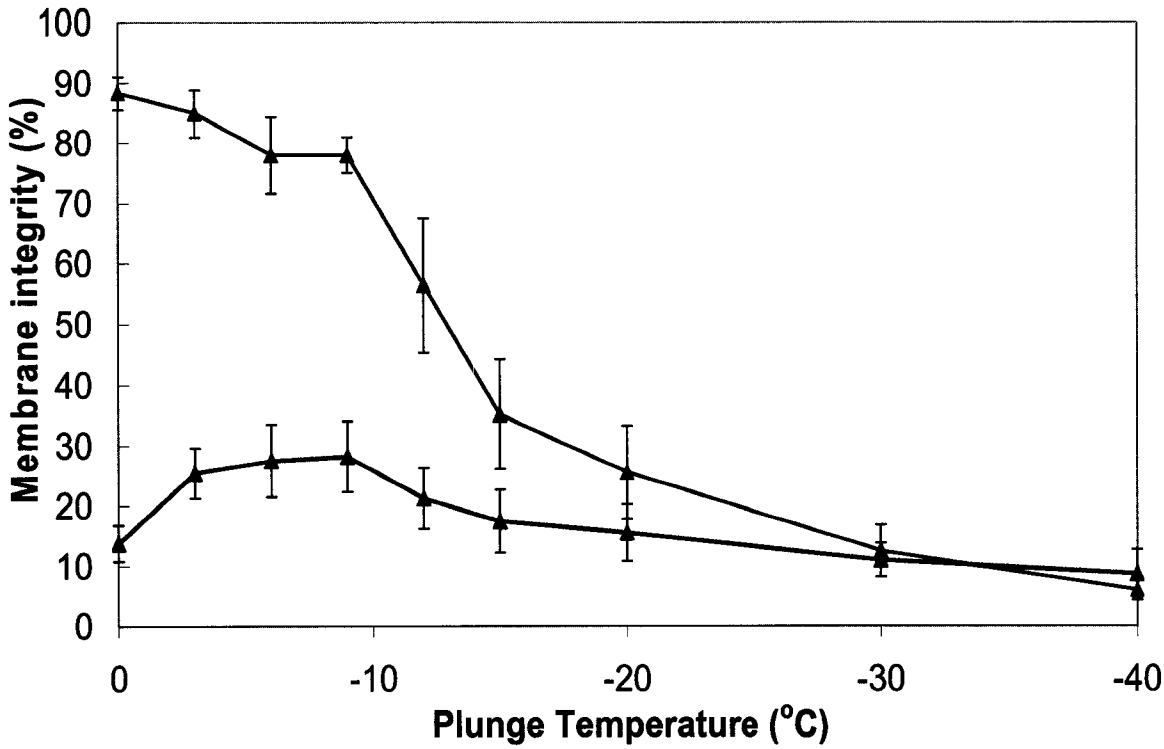

Figure 4-3. Membrane integrity of TF-1 cells (±SEM) in serum-free RPMI media after being cooled at 0.5°C/min to various subzero plunge temperatures and either thawed directly (upper curve) or plunge into liquid Nitrogen (lower curve) prior to thawing.

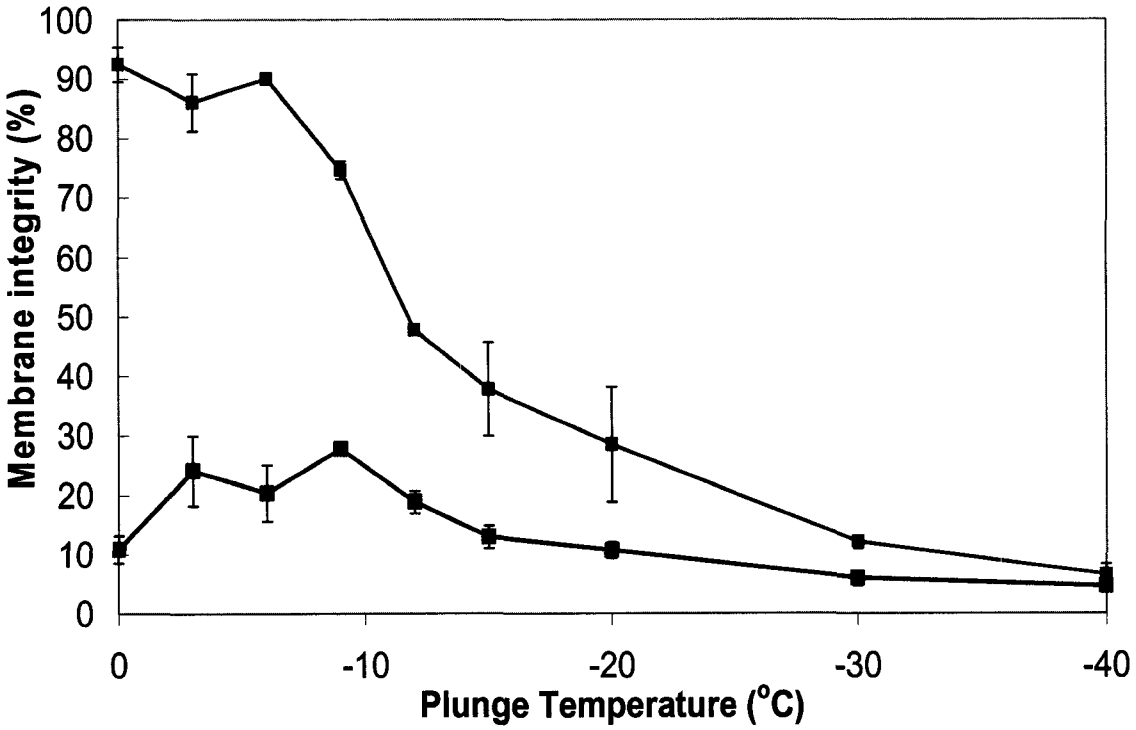

Figure 4-4. Membrane integrity of TF-1 cells (±SEM) in serum-free RPMI media after being cooled at 0.9°C/min to various subzero plunge temperatures and either thawed directly (upper curve) or plunge into liquid Nitrogen (lower curve) prior to thawing.

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Chapter 5: Theoretical design of a cryopreservation protocol

5.1 Introduction

The cellular responses to the formation of ice in surrounding solution are largely dependent on the movement of water across the plasma membrane. Ice formation causes osmotic stress on the cell membrane forcing water out of the cell to maintain equilibrium with the extracellular solution. The properties of the cell membrane, specifically the osmotic parameters, govern these changes in cell volume. The osmotic parameters can be used in simulations to theoretically model cellular responses to low temperatures. Simulations also provide precise results regarding changes in cell volume and the amount of supercooling. These results can then be used for comparisons between cryopreservation protocols and for comparison between different cell types which may be present in one tissue. Ultimately, simulations allow for unlimited theoretical protocols to be explored by controlling cooling and warming rate, plunge temperatures, and the components of the intracellular and extracellular compartments for any cell type for which the osmotic parameters are known.

To distinguish between the two types of injury, solution effects and intracellular ice formation, we simulated the empirical procedure of two-step freezing. The two-step freezing technique was developed by Farrant et al. and has provided a logical method to examine the effects of freezing injury on cell recovery due to non-linear cooling rates and to exposure to a range of subzero temperatures [2]. In their procedure, samples were cooled at an uncontrolled

non-linear cooling rate to various subzero plunge temperatures by being transferred to a preset bath before being 1) thawed directly from that holding temperature or 2) plunged to 196°C before thawing. McGann and Farrant later reported the subzero plunge temperatures and the length of hold time at that temperature were important variables to consider when attempting to maximize cell survival [9].

The objective of this study was to further our understanding of the theoretical responses of TF-1 cells, a model for hematopoietic stem cells (HSC) [5-7], to subzero plunge temperatures and to hold times at those temperatures. Simulations were done using the osmotic parameters of TF-1 cells reported in Chapter 2. The objective of these simulations was to theoretically determine the conditions of TF-1 cells at various stages of a freezing protocol. Maximum levels of intracellular electrolyte concentrations ($[KCl]_i$) and of supercooling were examined upon cooling the cells to -40°C, as indicators for solution effects injury and intracellular ice formation injury, respectively.

5.2 Simulations of two-step freezing protocol

Methods

Simulations were performed according to those done in Chapter 3 using the osmotic parameters of TF-1 cells (Table 2.3) in the *CryoSim5* program (Dr. Locksley McGann, University of Alberta, Canada). The simulations were based on a two-step freezing technique, which has been used to examine the effects of high solute concentrations and intracellular ice formation on cell survival during freezing [3]. The cryopreservation protocol was defined by assigning a starting

temperature and then varying the cooling rates, based on typical two-step freezing procedures. Supercooling and $[KCl]_i$ were used as indicators of potential intracellular ice formation and solution effects, respectively.

Temperature profiles

The two-step freezing technique involved rapidly cooling the samples to various subzero plunge temperatures before either being thawed directly in a 37°C water bath or plunged into liquid nitrogen first and then thawed [8]. For the simulations, cells were cooled using a temperature profile derived from Fourier's Law. Fourier's Law describes the rate of heat transfer which depends on the temperature distribution of the system, previously described in Chapter 3 [4]. The fitting constant was determined by monitoring the cooling profile of a sample taken from room temperature and exposed to the experimental subzero temperature with a Type T thermocouple (Omega, Laval, Canada). Figure 5-1 is a representative cooling profile of a sample cooled from room temperature to -15°C. This profile was then fitted to a curve and the equation was then used in simulations. The variations between the experimental and fitted curves were due to the latent heat of fusion.

Simulations were performed in which cells with no cryoprotectant were cooled to various subzero plunge temperatures ranging from -3°C to -40°C and held at that temperature for 0.3, 0.5, 0.7, 1, 2, 3, 5, 7, or 10 minutes, prior to being plunged to -40°C (325°C/min) [1].

5.3 Results

Changes in cell volume during cooling

Figure 5-2 shows simulation results of temperature as a function of time for TF-1 cells cooled to various subzero plunge temperatures ranging from -3°C to -30°C, held for various hold times (minutes), prior to being plunged to -40°C (325°C/min). Based on the results from the simulations, the hold times were grouped according to similarities of changes in cell volume, $[KCl]_i$, and supercooling: hold times of 1 minute or less will be represented by the 0.5 minute data; hold times between 2 and 5 minutes will be represented by the 3 minute data; and hold times of between 7 and 10 minutes will be represented by the 10 minute data. Figure 5-3 demonstrates the changes in cell volume as a function of temperature upon cooling to various subzero plunge temperatures ranging from -3°C to -30°C, held for a duration, prior to being plunged to -40°C (325°C/min). The data shown is for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min. hold times. Cells showed a progressive decrease in cell volume upon cooling. Cells only held for 0.5 minutes at the subzero temperature did not reach the same volumes as those held for 3 or 10 minutes at -3°C and -35°C. This data suggests that the cells have not had sufficient amount of time to dehydrate with a hold time of 0.5 minutes, as opposed to greater than 3 minutes, for both high and low subzero plunge temperatures. This data also indicates that the cells would have a higher amount of supercooling at these outlying plunge temperatures due to the lack of cellular dehydration. Also, with lower concentrations of $[KCl]_i$, it is possible that the cells would not be subjected to high solution effects.

Supercooling during cooling

Figure 5-4 demonstrates the changes in supercooling as a function of temperature upon cooling to various subzero plunge temperatures ranging from -3°C to -30°C, prior to being plunged to -40°C (325°C/min). Data shown are for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min. hold times. Supercooling of up to 10°C occurs for all the hold times down to -12°C. This suggests that supercooling plays a key role in potential injury during freezing to lower subzero plunge temperatures. At these lower temperatures, cells were exposed to increasingly supercooled conditions up to 30°C at -30°C.

$[KCl]_i$ during cooling

Figure 5-5 demonstrates the changes in $[KCl]_i$ as a function of temperature upon cooling to various subzero plunge temperatures ranging from -3°C to -30°C, prior to being plunged to -40°C (325°C/min). The data shown is for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min. hold times. Cells cooled to lower subzero plunge temperatures showed increasing concentrations of $[KCl]_i$, with the highest concentration for cells cooled to -30°C and held for greater than 3 minutes. This correlates with the gradual decrease in cell volume reported in the previous section. This gradual increase in $[KCl]_i$ demonstrates the potential for increased solution effects upon cooling to the lower subzero plunge temperatures. The data show similar concentrations of $[KCl]_i$ at all plunge temperatures except at -3°C and below -30°C.

Maximum supercooling and $[KCl]_i$ during cooling

The maximum amount of supercooling was calculated as the highest amount of supercooling which occurred throughout the cooling profile for each plunge temperature. Figure 5-4a supercooling as a function of temperature for TF-1 cells with arrows indicating where the maximum supercooling was determined for the various plunge temperatures. The maximum amount of supercooling was calculated and graphed as a function of plunge temperature (Figure 5-6). Data is shown for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min. hold times. The maximum supercooling obtained appears to be the primary contributor to potential injury, which suggests that a target plunge temperature between -6°C to -12°C would lead to high levels of survival because the supercooling does not exceed 10°C. Cells held for 0.5 minutes have a more narrow range of optimal plunge temperatures, limited by the amount of supercooling. These results correlate with the lack of cellular dehydration discussed in the previous sections.

The maximum amount of $[KCl]_i$ was calculated as the highest concentration of KCl which occurred throughout the cooling profile for each plunge temperature. Figure 5-5a shows the $[KCl]_i$ as a function of temperature for TF-1 cells, with arrows indicating where the maximum $[KCl]_i$ was determined for the various plunge temperatures. The levels of maximum $[KCl]_i$ for cells held for 0.5, 3 and 10 minutes gradually increase from -3°C to -20°C (Figure 5-6). The slope between -3°C and -6°C varies from cells held for 0.5 minutes and 3 to 10 minutes, suggesting that at plunge temperatures between -3°C and -6°C,

there may be a difference in cell recovery between 0.5 minutes and 3 to 10 minutes. For all the hold times, based on the temperature range set by the 10°C limit to supercooling, the data suggests that the lower $[KCl]_i$ levels would result in better cell recovery. Figure 5-7 shows the plunge temperature ranges for cells held for 3 minutes based on 10°C supercooling and 3 M $[KCl]_i$. This range varies between 0.5 minute hold time and the 3 and 10 minute hold time. However, a target plunge temperature of approximately -6°C should result in the highest cell recovery for all the hold times.

These simulations suggest that supercooling plays a key role in two-step freezing and the effects of increasing solute concentrations are secondary. The optimal temperature for plunging the cells after the initial cooling phase is a function of the amount of time spent to cool to a specific temperature, which influences $[KCl]_i$ and supercooling.

5.4 Protocol Design

Based on the simulations using two-step cooling profiles, the amount of supercooling indicates that intracellular ice formation plays a key role in the freezing injury for TF-1 cells held for all of the hold times. However, the range of plunge temperatures which have greater than 10°C supercooling differs somewhat for the hold times. For cells held for less than 1 minute, represented by 0.5 minutes data, maximum supercooling is within the 10°C limit between plunge temperatures of -4°C and -12°C. For cells held for greater than 2 minutes, represented by 3 minutes, supercooling is with the 10°C limit between plunge temperatures of -4°C and -12°C. Also, based on the simulations, solution

effects are proposed to play a role in freezing injury to TF-1 cells as well. The minimum levels of $[KCl]_i$ are at the higher subzero plunge temperatures within the range set by the 10°C supercooling limit. Therefore, the data suggests that there would be a peak to cell survival at the higher plunge temperatures within the range as well. Based on the comparison of theoretical and experimental data in Chapter 4, we can predict that cells cooled to -12°C and below, where the $[KCl]_i$ is approximately 3 M, will be damaged due to solution effects and supercooling.

Although, the plunge temperature ranges were the same for 2 to 5 minutes and 7 to 10 minutes hold times, we would propose that freezing cells using 2 to 5 minutes may show a significant increase in cell survival due to decreased exposure time to solutes as compared to the longer hold times. A shorter hold time, may result in increased supercooling, as seen in these simulations; conversely a longer hold time, may result in increased exposure time to high solute concentrations. The optimal hold time should only allow sufficient time for the cell to dehydrate, while minimizing exposure to solutes. Empirical assessment would be required to further differentiate between the effects of subzero temperature and hold times.

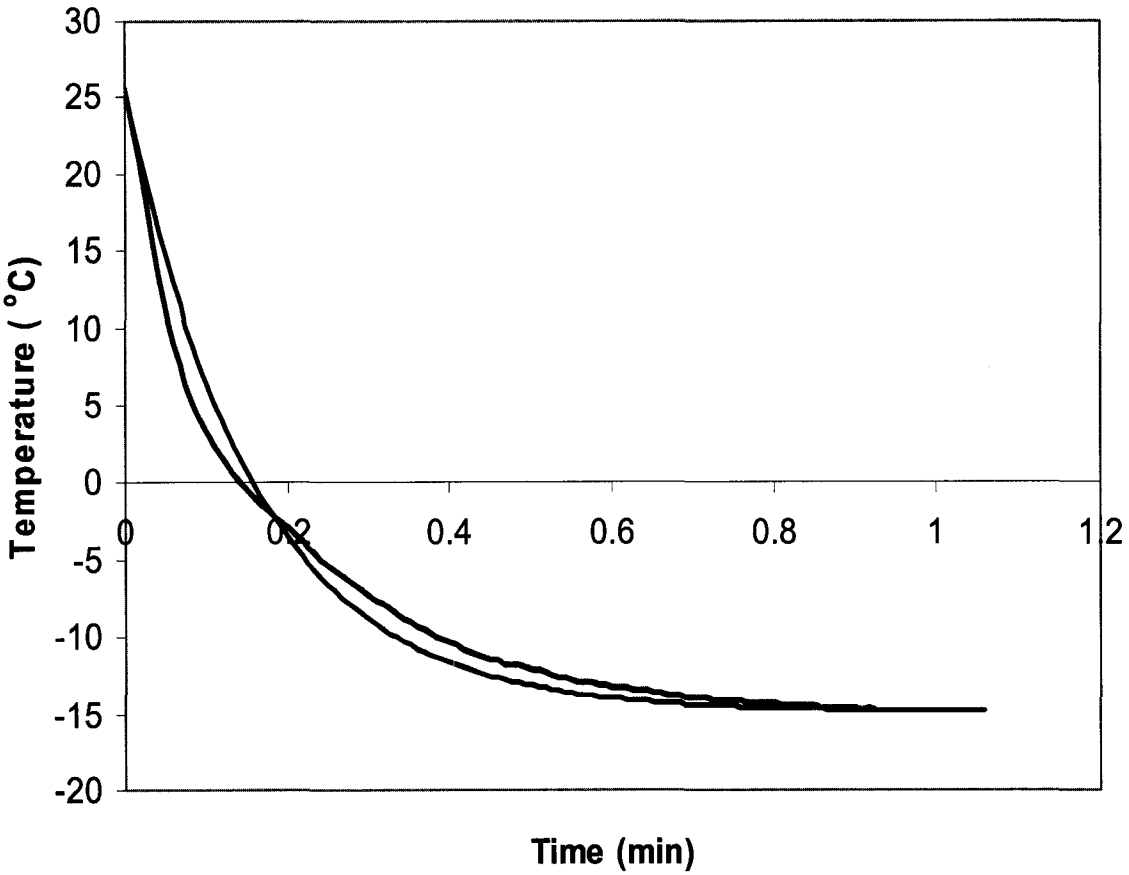

Figure 5-1. A representative cooling profile for TF-1 cells cooled from room temperature to -15°C measured with a thermocouple (measured). The curve is fitted according to Fourier's Law (fitted).

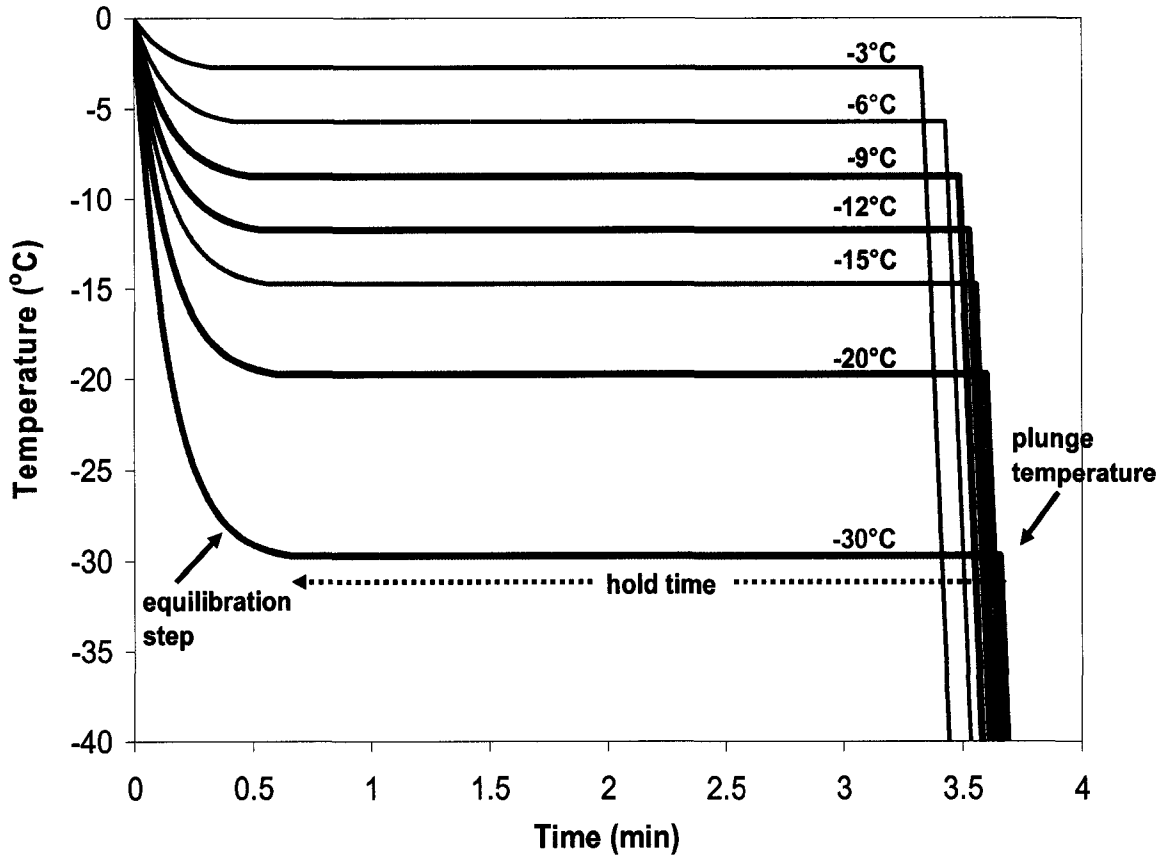

Figure 5-2. Simulations of temperature as a function of time for TF-1 cells cooled to various subzero plunge temperatures (-3°C to -40°C) using Fourier's Law (constant=7) and held for 3 minutes, prior to being plunged to -40°C (325°C/min).

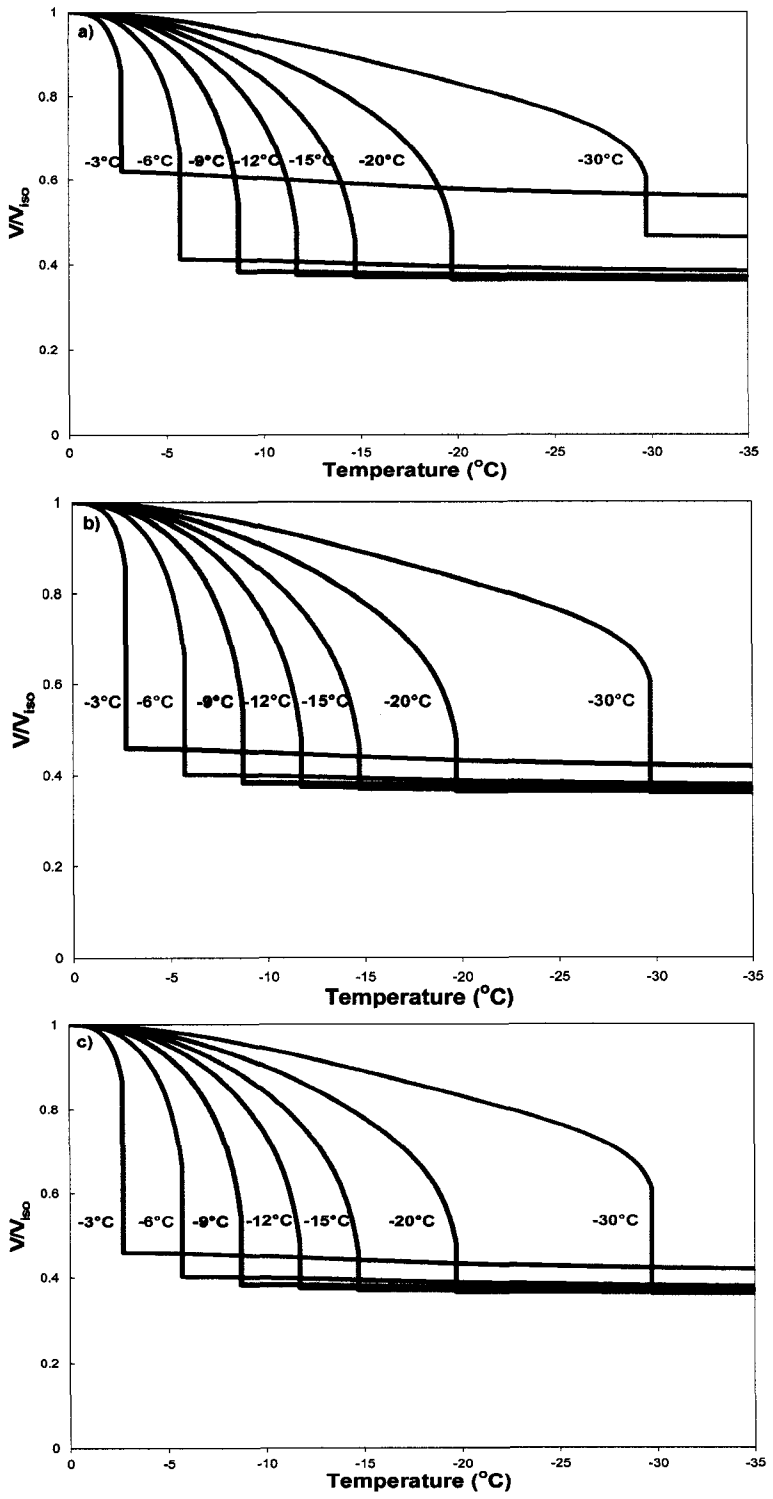

Figure 5-3. Volume as a function of temperature for TF-1 cells using Fourier's Law (constant=7) to various subzero plunge temperatures (-3°C to -40°C) and held at that temperature for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min., prior to being plunged to -40°C (325°C/min).

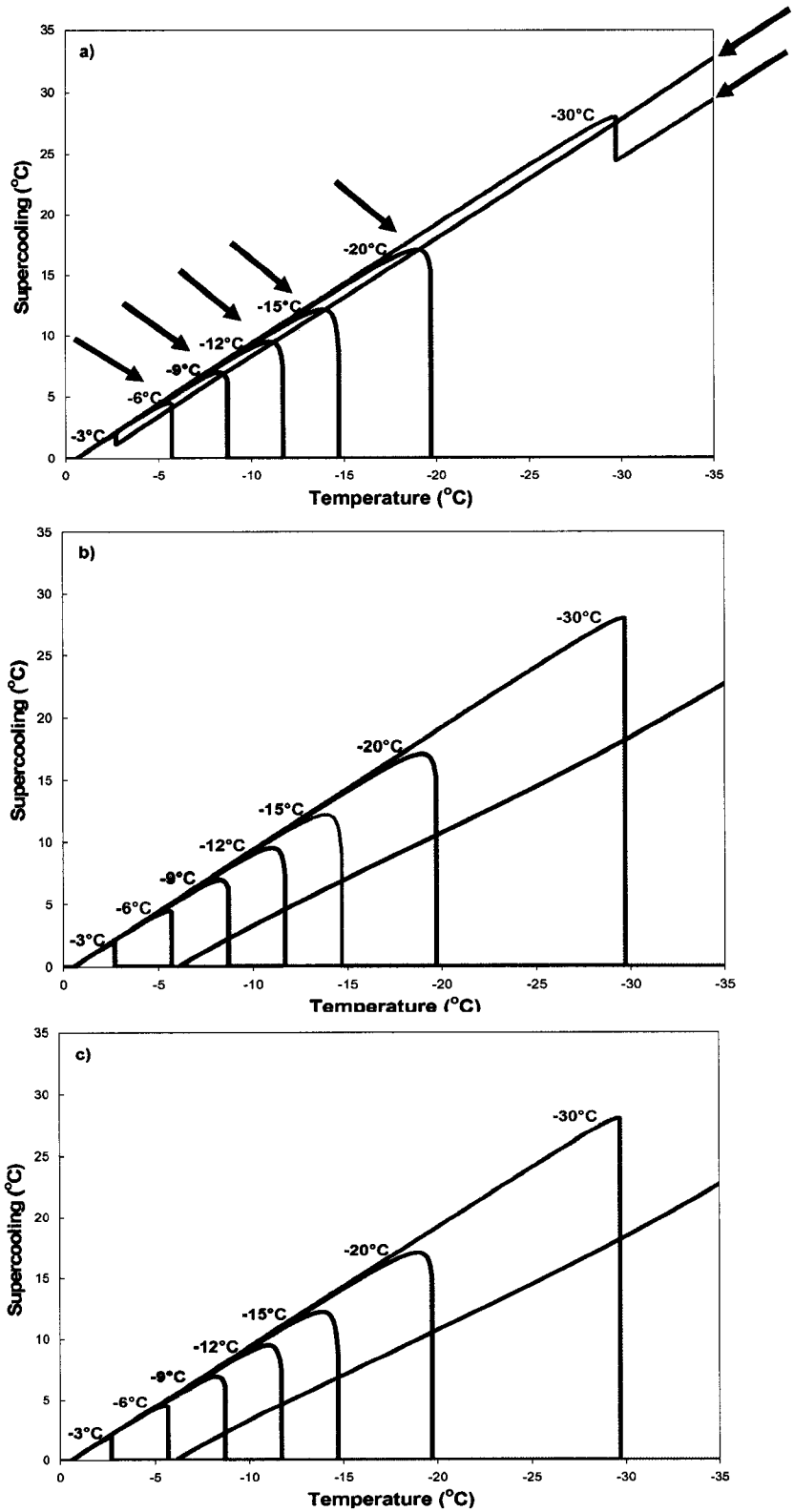

Figure 5-4. Supercooling as a function of temperature for TF-1 cells cooled to various subzero plunge temperatures (-3°C to -40°C) and held at that temperature for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min., prior to being plunged to -40°C (325°C/min). Arrows indicate maximum supercooling.

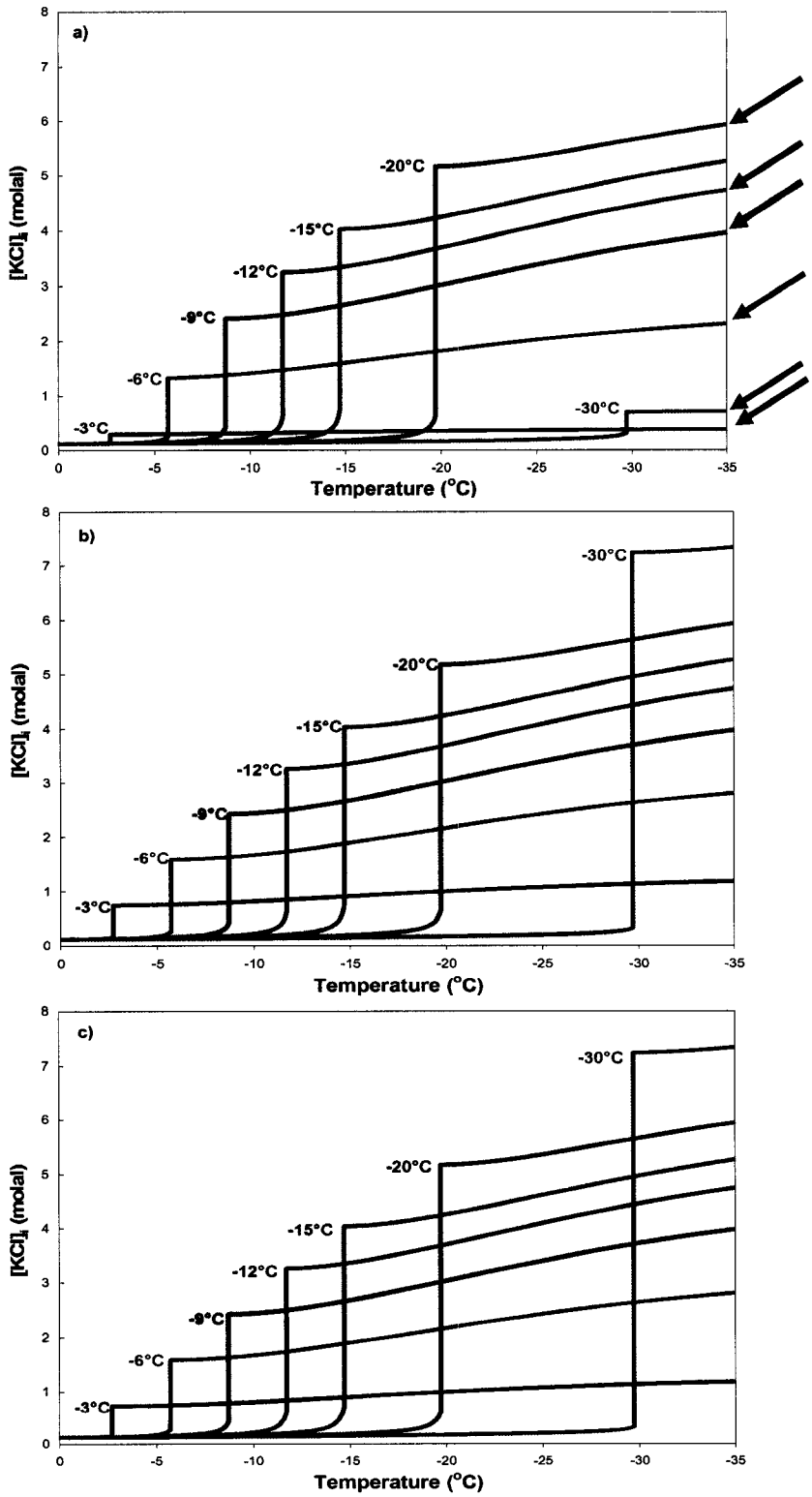

Figure 5-5. $[KCl]_i$ as a function of temperature for TF-1 cells using Fourier's Law to various subzero plunge temperatures (-3°C to -40°C) and held at that temperature for (**a**) 0.5 min., (**b**) 3 min., and (**c**) 10 min., prior to being plunged to -40°C (325°C/min). Arrows indicate maximum $[KCl]_i$.

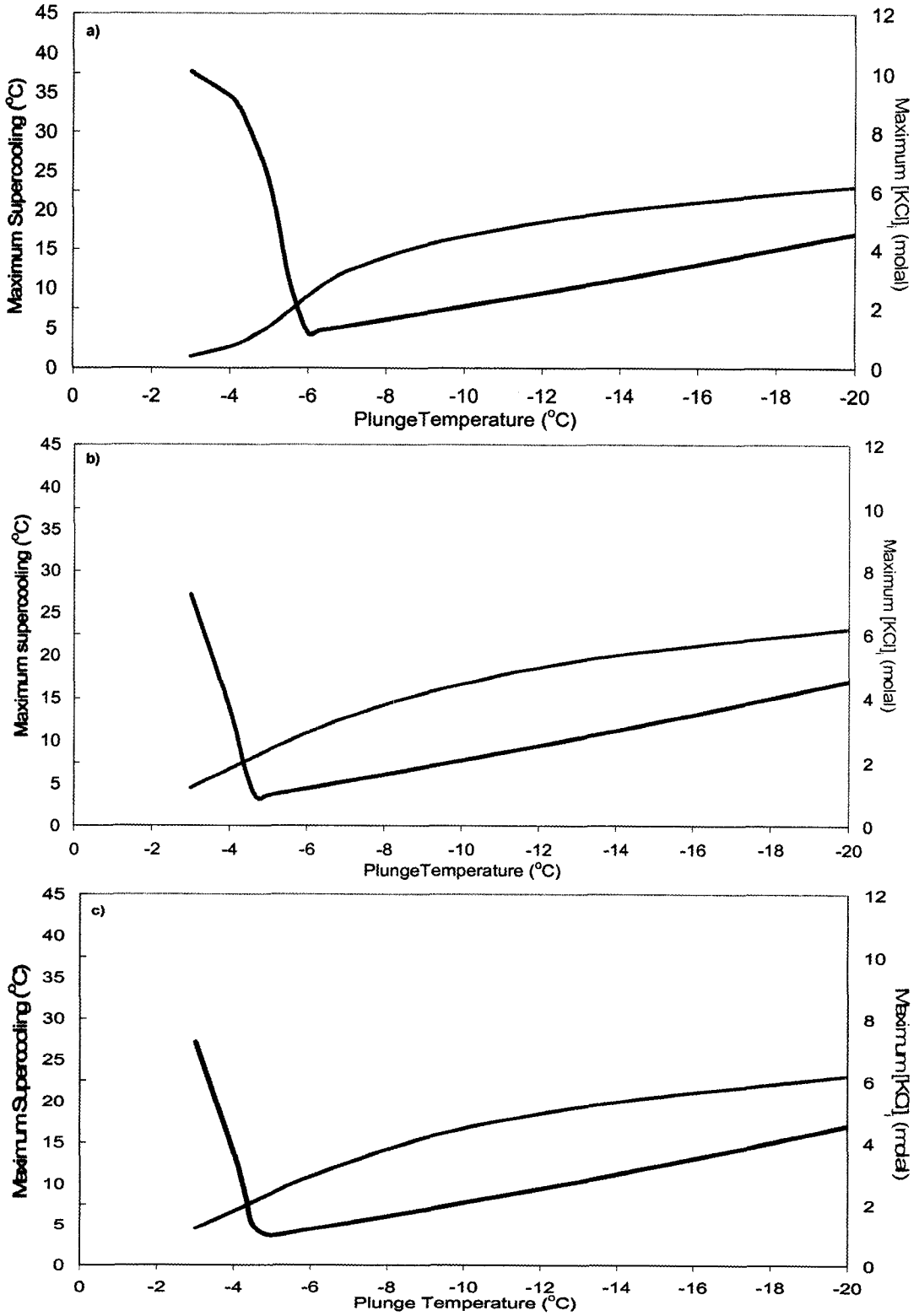

Figure 5-6. Maximum $[KCl]_i$ and maximum supercooling as a function of temperature for TF-1 cells cooled to various subzero plunge temperatures and held at that temperature for 0.5 min. (**a**), 3 min. (**b**), and 10 min. (**c**), prior to being plunged to -40°C (325°C/min).

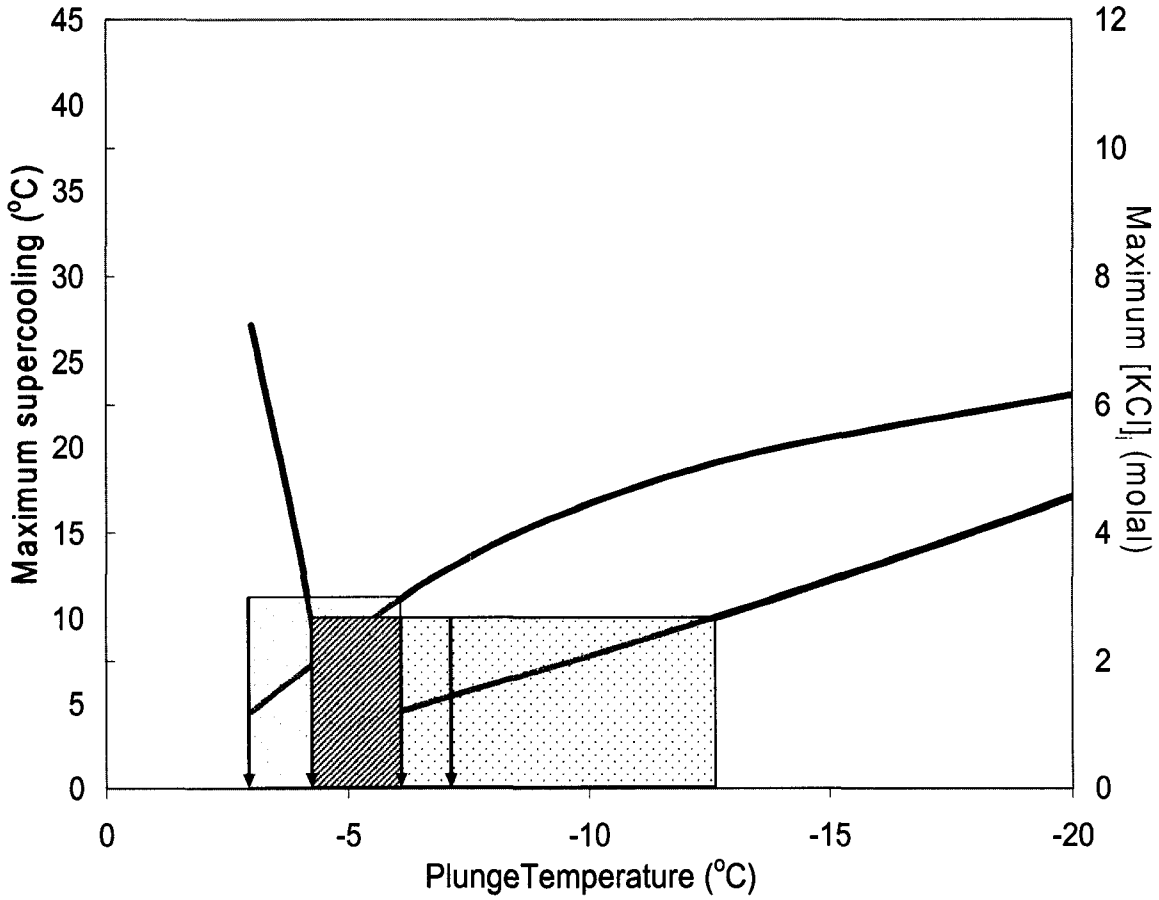

Figure 5-7. Maximum $[KCl]_i$ and maximum supercooling as a function of temperature for TF-1 cells cooled to various subzero plunge temperatures and held at that temperature for 3 min. prior to being plunged to -40°C (325°C/min). The shaded box indicates the optimal plunge temperature of between -4°C and -6°C based on 10°C supercooling and 3 M $[KCl]_i$.

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Chapter 6: Experimental correlation and optimization of a theoretically-designed cryopreservation protocol

6.1 Introduction

Traditionally, cryopreservation protocols are determined empirically, by varying the cooling rates, type of cryoprotectant and concentration of cryoprotectant. In cryopreservation procedures, cells are cooled at a finite rate which is optimized for the cell type and cryoprotectant. Optimization can be performed empirically using not only graded freezing [14], but the two-step freezing technique as well [6]. In order to explore the two types of injury proposed in the 'two-factor hypothesis' [12], Farrant et al. developed the two-step freezing technique, which sought to explore the two types of freezing injury by determining the subzero temperature range where damage occurs [6]. The samples were cooled from room temperature to various subzero plunge temperatures before being either thawed directly or plunged into liquid nitrogen first and later thawed.

The simulations performed in Chapter 5 predicted that subzero plunge temperature and time spent at that temperature were critical variables in the optimization of cryopreservation protocols. In order for simulations to be used in cryopreservation, it is necessary to test the predictions of simulations empirically. The purpose of this study was to explore the range of subzero plunge temperatures and time spent at those temperatures. Two-step freezing experiments were conducted with TF-1 cells and compared with the cell survival

outcomes that were theoretically predicted in Chapter 5. Membrane integrity was used as an assay for freeze-thaw injury. Membrane integrity has been used as an indicator of cell damage during freezing, as it has been shown that the membrane is a site of freezing-thawing injury [1]. Also, it has been shown that there is a correlation between intracellular freezing and membrane damage for cells in suspension [1,11].

6.2 Materials & Methods

TF-1 cell culture

TF-1 cells (ATCC, Manassas, Virginia) were grown at 37°C in 5% CO_2 in RPMI 1640 Medium Modified (ATCC) with 10% fetal bovine serum (FBS) (ATCC), and supplemented with 2 ng/mL recombinant human GM-CSF (Stemcell Technologies, Vancouver, Canada). Cells were maintained between 0.1 x 10^6 and 1 x cells/mL, according to ATCC guidelines. Prior to experiments, cells were washed twice with serum-free RPMI media and incubated overnight. Cells were then centrifuged and re-suspended at a concentration of 4 x 10^6 cells/mL, which was necessary for the viability assessment program to be used.

Experimental solutions

TF-1 cells were re-suspended in serum-free RPMI prior to the two-step freezing experiments.

Two-step freezing experiments

Samples of 0.2 mL cell suspension, in serum-free RPMI, in glass tubes were allowed to equilibrate at room temperature for 5 minutes. Control samples were either warmed in a 37°C water bath or plunged into liquid nitrogen.

Experimental samples were individually transferred into a methanol bath preset at -3, -6, -9, -12, -15, -20, -30, and -40°C and allowed to equilibrate for 2 minutes at that temperature prior to ice nucleation with cold forceps. After nucleation, samples were allowed to equilibrate for 3 minutes before either being thawed directly in a 37°C water bath or plunged into liquid nitrogen. Samples were kept in liquid nitrogen for a minimum of 1 hour prior to being thawed in a 37°C water bath. Duplicate samples were used for both the direct thaw and the plunge conditions at each plunge temperature. Each experiment was repeated in triplicate.

The two-step freezing experiments were repeated with varying hold times. Cells were cooled to -5, -7, -9, -12, -15, and -25°C and allowed to equilibrate for 2 minutes prior to ice nucleation with cold forceps. After nucleation, samples were allowed to equilibrate for 0.5 or 10 minutes before either being thawed directly in a 37°C water bath or plunged into liquid nitrogen. Samples were kept in liquid nitrogen for a minimum of 1 hour prior to being thawed in a 37°C water bath. Duplicate samples were used for both the direct thaw and the plunge conditions at each plunge temperature. Each experiment was repeated in triplicate.

Viability assessment

Cell viability was assessed by a membrane integrity assay. The assay was performed by incubating cells with SYTO® 13 (Molecular Probes, Eugene, Oregon) and ethidium bromide (EB) (Sigma, Mississauga, Canada) [18]. Syto 13 permeates the cell membrane of all cells and complexes with DNA and it

fluoresces green under UV exposure. EB penetrates cells with a damaged plasma membrane and also complexes with DNA fluorescing red under UV conditions. The dual stain allows for differentiation between cells with and without intact plasma membranes.

The Syto/EB stain was prepared using 40 μL of 2.5 mM EB stock solution and 10 μL of 5 mM Syto® 13 stock solution mixed with 350 μL 1X phosphate-buffered saline (PBS). Final concentrations were 0.25 mM EB and 0.125 mM Syto. Twenty μL of stain was added to each sample and allowed to incubate for 2 minutes at room temperature. Fluorescent images were captured using a Leitz Dialux 22 fluorescence (440-480 nm) microscope (Leitz, Germany) fitted with a PIXERA DiRactor (Pixera Corporation, Los Gatos, CA, USA) digital camera. The Viability Assessment Program (The Great Canadian Computer Company, Spruce Grove, Canada), which counts red versus green pixels was used to quantify cell membrane integrity from digital images [8]. This method measures membrane integrity of the cell remaining after experimental treatment.

6.3 Results

Varying plunge temperature

TF-1 cells were suspended in serum-free RPMI to various plunge temperatures up to -40°C and held for 3 minutes, prior to being thawed directly or plunged into liquid nitrogen (Figure 6-1). Overall, cells demonstrated a higher percentage of membrane integrity than cells cooled at 0.2°C/min to 0.9°C/min presented in Chapter 4. Cells plunged into liquid nitrogen showed comparable results for membrane integrity to cells directly thawed from temperatures ranging

from -15°C to -40°C. Cells thawed directly from the plunge temperatures showed a 50 % decrease in membrane integrity by -17°C, indicating that a major portion of cells were damaged prior to being plunged into liquid nitrogen (Figure 6-1). However, this membrane damage occurred at a higher subzero plunge temperature for cells cooled at 0.9°C/min. The maximum recovery of 58.8±6.5% was seen for TF-1 cells cooled to -12°C or -15°C before being plunged into liquid nitrogen. These results were higher than those previously reported in Chapter 4 of approximately 28.2%. Furthermore, these results were comparable with those shown for cooling at 0.9°C/min in 10% DMSO/RPMI of 63.7% (Chapter 4).

Varying experimental hold time

TF-1 cells were cooled to -5, -7, -9, -12, -15, or -25°C and allowed to equilibrate for 2 minutes prior to ice nucleation with cold forceps. After nucleation, samples were allowed to equilibrate for varying times (0.3, 0.5, 0.7, 1, 2, 3, 5, 7, and 10 minutes) before either being thawed directly in a 37°C water bath or plunged into liquid nitrogen. Figure 6-2a shows the membrane integrity of TF-1 cells as a function of hold time for cells cooled to -5°C, held and plunged into liquid nitrogen. Comparable results of membrane integrity were obtained for cells held at -7°C to -9°C (results not shown). However there was minimal membrane integrity for cells held below -25°C (data shown in Figure 6-2b). Cells that were directly thawed from the subzero plunge temperatures after being held showed progressive decrease in membrane integrity based on reduced temperature and increased duration of hold time (Figure 6-3a). Results indicated a high percentage of membrane integrity of 55 to 60%, when cells were held for 1-5

minutes at high subzero plunge temperatures. Cooled cells from room temperature to -5°C and -7°C and held for 1-3 minutes, prior to plunging into liquid nitrogen resulted in the highest percentage of membrane integrity of approximately 60% (Figure 6-3b). A hold time of greater than 5 minutes resulted in a marked decrease in cell survival. This data indicates that there is a zone of subzero plunge temperatures (-5°C to -15°C), when held for 1-3 minutes, which confers protection against injury comparable to DMSO.

6.4 Correlation with theoretically-designed protocol

Discussion of experimental results

The experimental results for cryopreserving TF-1 cells without cryoprotectants indicate that cells can be cryopreserved without DMSO. This data indicates that there is a zone of subzero plunge temperatures (-5°C to -15°C), when held for 1-3 minutes, which confers comparable protection against injury to the standard 10% DMSO/RPMI solution, previously reported in Chapter 4. This range would constitute an optimal subzero temperature range for these hold times based on experimental results.

Comparison of theoretical and experimental results

Simulations were done based on an empirical approach to cryopreservation, two-step freezing, which can be used to examine the role of exposure to subzero plunge temperatures and exposure time. The cooling rates used in the two-step freezing protocol are governed by Fourier's Law and were determined experimentally in Chapter 5. Cells were exposed to increasingly supercooled conditions up to 40°C at a plunge temperature of -40°C.

Supercooling appears to be the primary contributor to potential freezing injury. This research supported the upper limit of 10°C supercooling, previously reported by Mazur. The proposed target plunge temperature was suggested to be between -4°C and -12°C, as supercooling was restricted to less than 10°C, which is comparable to the range determined empirically. Also, based on levels of intracellular KCl ($[KCl]_i$), it was suggested that the higher subzero plunge temperature would have the lowest potential for solution effects based on the lack of cell dehydration, which was also supported by this data.

Two-step freezing experiments demonstrated a high percentage of membrane integrity for TF-1 cells when cells were cooled to between -5°C and -12°C and held for 1-5 minutes. These plunge temperatures corresponded with the theoretical values of 5°C to 10°C supercooling, which suggested that a certain amount of supercooling is necessary to achieve a higher viability [3]. However, this also supports the belief that excessive supercooling may lead to damage as a result of intracellular ice formation.

Based on the simulations, the duration of time the cells were held at the subzero plunge temperature was also considered an important factor. When cells were held for 0.5 minutes, they did not have sufficient time to dehydrate and reach the same volume as cells held for greater than 2 minutes. This excess intracellular water may have caused damage by forming ice upon subsequent cooling. According to the two-step freezing experiments, cells held for 2 minutes at -5°C and for 5 minutes at -12°C, had the highest cell recovery. Those held for 10 minutes may have been exposed to high concentrations of solutes for a

duration which was damaging. Simulations from Chapter 5 predicted that there was no difference in $[KCl]_i$ concentrations and supercooling between hold times down to -25°C. The experimental results demonstrated that the differences in membrane integrity between the hold times may depend on the duration of exposure, which is consistent with the theoretical results.

For all the hold times, simulations predicted a progressive increase in $[KCl]_i$ upon cooling to lower plunge temperatures down to -25°C for cells held for 0.5 minutes. The experimental results demonstrated a progressive decline in membrane integrity for cells thawed directly from subzero plunge temperatures. At low subzero plunge temperatures (<-20°C), cells directly thawed had low percentages of membrane integrity (<30%). Therefore, either the exposure time and/or the concentration of solutes may have been significant variables for freezing injury. The results of this study are based on damage to the cell membrane, however if the ultimate goal is to determine the optimal cryopreservation protocol for HSCs, than the multipotentiality of these cells would have to be analyzed using colony assays [11,14,19].

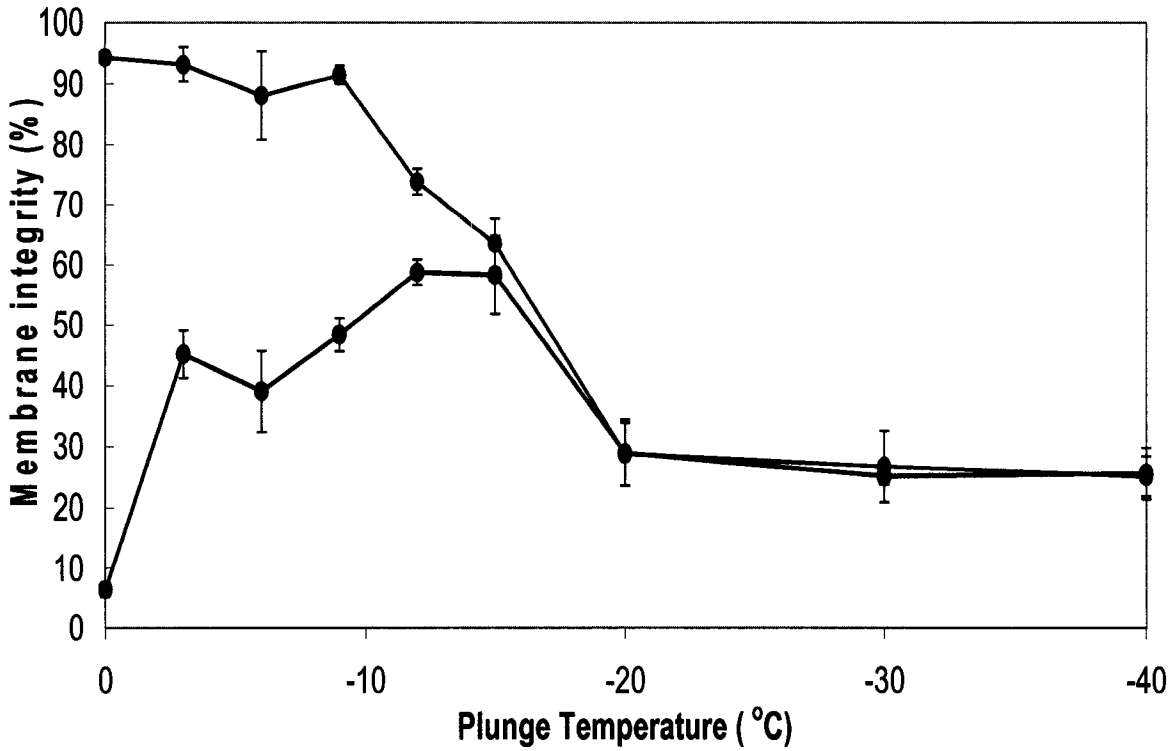

Figure 6-1. Membrane integrity for TF-1 cells (±SEM) in serum-free RPMI media cooled to various subzero plunge temperatures from room temperature, held 3 minutes, and then either thawed directly (upper curve) or plunged into liquid nitrogen (lower curve) before being thawed.

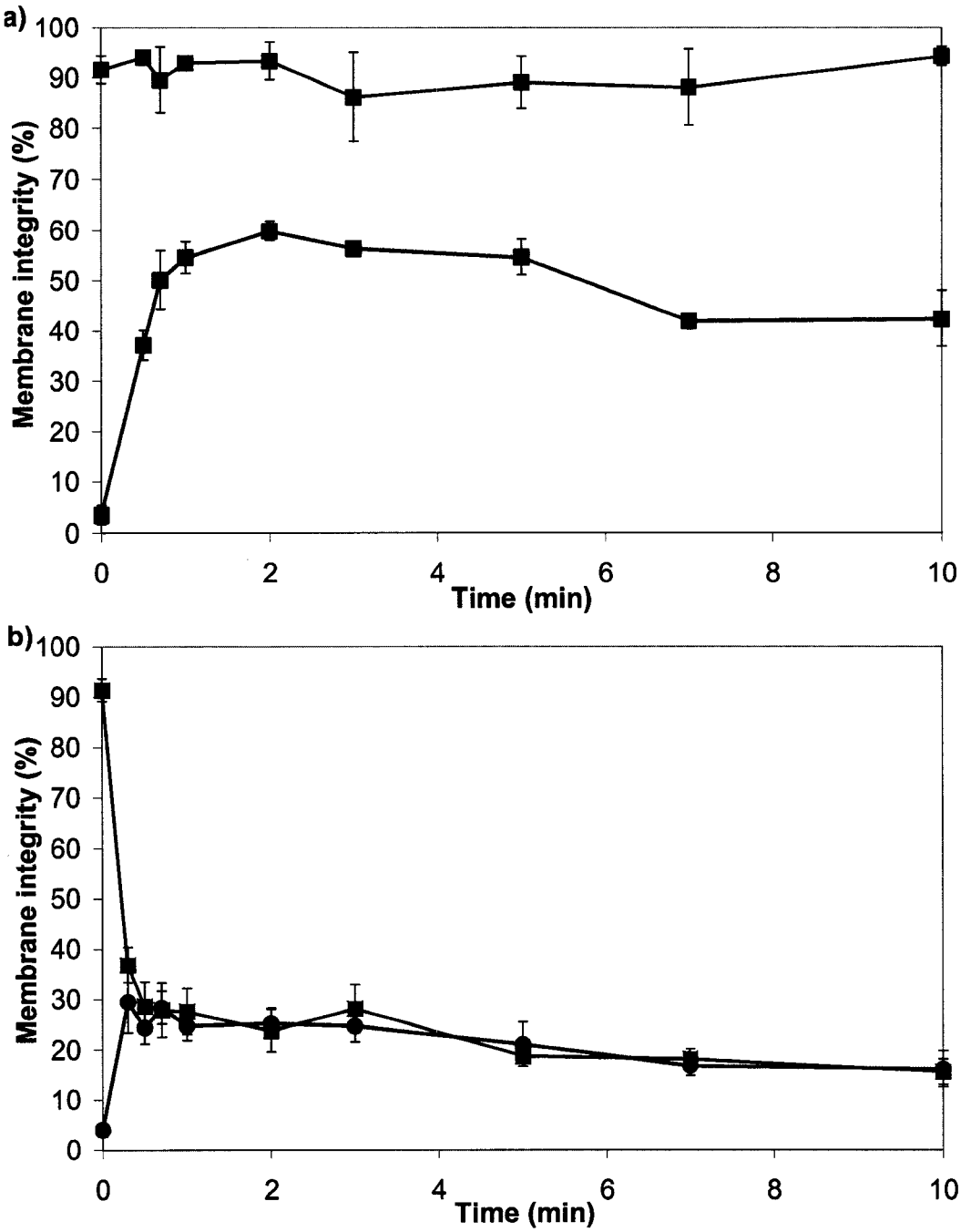

Figure 6-2. The membrane integrity of TF-1 cells (±SEM) in serum-free RPMI media as a function of hold time for cells cooled to (**a**) -5°C and (**b**) -25°C, held for a period of time before either thawed directly (upper curves) or plunged into liquid nitrogen (lower curves) before being thawed.

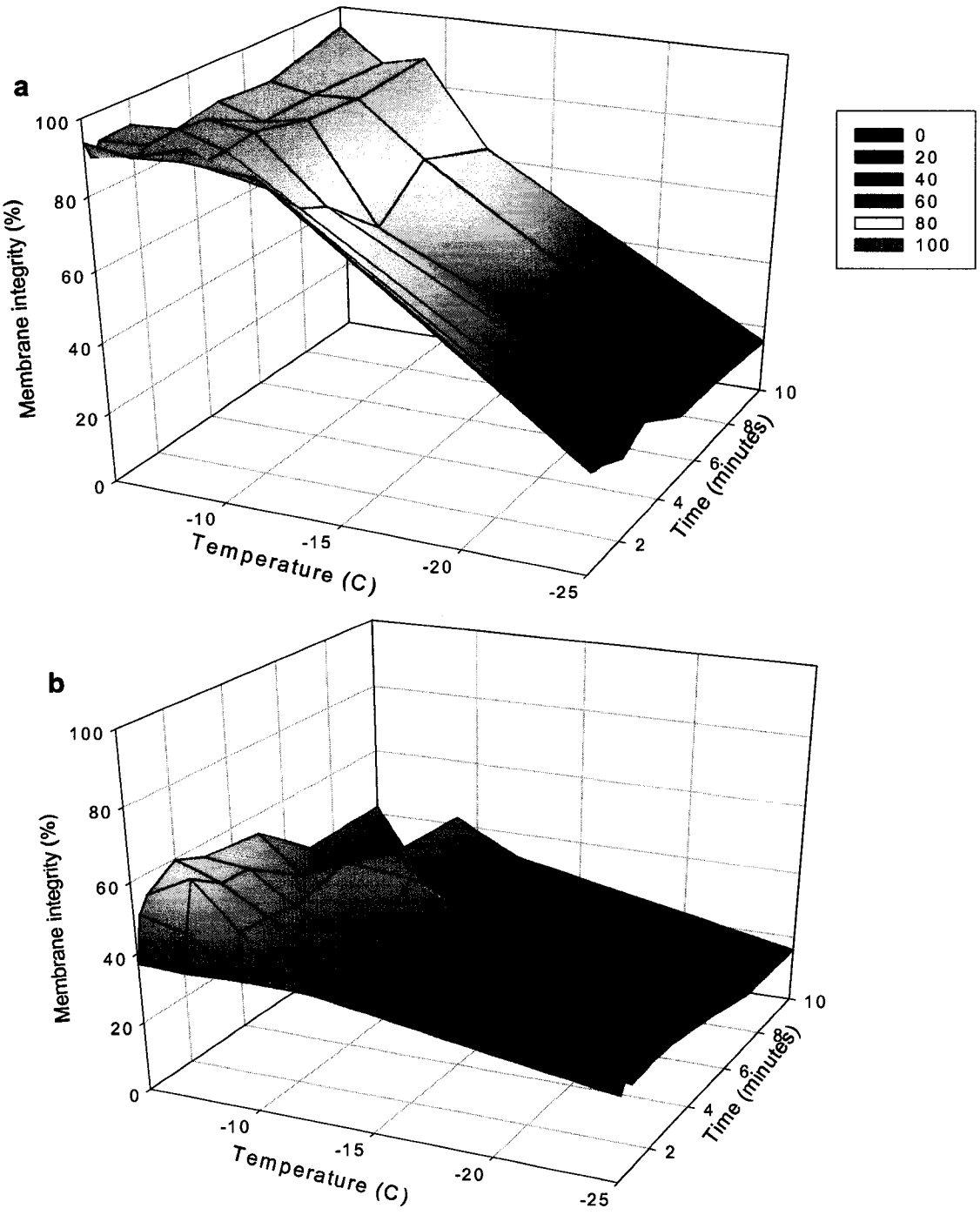

Figure 6-3. Contours of membrane integrity of TF-1 cells in serum-free RPMI media after being cooled to various subzero plunge temperatures and held for a duration ranging from 0.5 to 10 minutes before either (**a**) thawed directly or (**b**) plunged into liquid nitrogen prior to thawing.

6.5 References

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Chapter 7. Overall Discussion and General Conclusions

7.1 Summary of thesis

Development of protocols for cryopreservation of cells has largely been approached empirically. Although this has met with success for many cellular systems, protocols may not be achieving the maximum recovery of cells. In the area of hematopoietic stem cell (HSC) preservation, current protocols use dimethyl sulfoxide (DMSO) as a cryoprotectant and a cooling rate of 1°C/min to achieve approximately 70% cell recovery. Adverse effects due to DMSO toxicity for patients undergoing HSC transplantation are a concern for transplant physicians. Cryobiologists face the challenge of maintaining cell viability at a range of subzero temperatures, including liquid nitrogen temperatures (-196°C), without the use of toxic cryoprotectants. Therefore, developing a cryopreservation protocol, which allows for the recovery of a high percentage of HSC from the frozen state without the use of toxic cryoprotectants, is a valid objective for applications in stem cell transplant medicine.

This study used the osmotic characteristics of TF-1 cells, as a model for hematopoietic stem cells, to theoretically explore the cell's response to low temperatures. Using thermodynamic equations, osmotic parameters were determined using a modified Coulter counter, fitted with a cell size analyzer. The cells were exposed to hypertonic solutions and their change in volume was monitored over time. Osmotic parameters from this study showed that TF-1 cells are relatively slow responding cells and that the movement of water across the plasma membrane is highly dependent on temperature.

The second study in this thesis was to simulate the responses of TF-1 cells to low temperatures typically involved in cryopreservation protocols. Low and high linear cooling rates (0.2°C/min to 100°C/min) were used in graded freezing protocols, to explore the potential of damage during freezing due to solution effects and intracellular ice formation. Previous work has demonstrated the use of supercooling, as an indicator for intracellular ice formation [4]. This research demonstrates the novel approach of using intracellular KCl concentration ($[KCl]_{i,}$) as an indicator for solution-effects damage [1]. Based on the results of these virtual experiments, we proposed that for low cooling rates, the length of time exposed to high solute concentrations would cause damage to the cell and for high cooling rates, supercooling leading to intracellular freezing, would also be damaging.

Application of Fourier's Law to define the cooling profiles is an example of simple thermodynamic applications to practical systems. Modeling the equilibration step at a constant temperature using Fourier's Law is a novel approach in cryobiology. This provides an accurate description of sample equilibration linking theory with experimental process.

The third study in this thesis correlated biological outcomes with theoretical modeling of cellular responses at low temperatures. The graded freezing procedure was used to examine the effects of slow cooling on membrane integrity. This research demonstrated that the longer the duration of exposure to high concentrations of solutes, ie. lower cooling rates, the more damage there was caused to the cell. In addition to cooling rates, subzero

temperatures and the duration of time spent at those temperatures were critical variables in minimizing cryoinjury.

The fourth study performed virtual experiments of the two-step freezing procedure. Non-linear cooling rates, based on Fourier's Law, to subzero temperatures and various hold times were used in two-step cooling protocols, to explore potential damage due to supercooling and solution effects [2]. Using high cooling rates produced a range of supercooling levels, which at greater than 10°C would likely cause intracellular ice formation [4], and $[KCl]_i$ levels, which greater than 3 M could be damaging [3]. The minimum levels of $[KCl]_i$ are at the higher subzero temperatures within the range set by the 10°C supercooling limit. This research demonstrated the use of simulations to define a range of subzero temperatures, and duration of exposure, which would minimize cryoinjury. Based on the comparison of theoretical and experimental data, it was possible to predict that cells cooled to -12°C and below, where the $[KCl]_i$ exceeds 3 M, will be damaged due to solution effects and supercooling.

The fifth study was based on the two-step freezing procedure as a tool to examine the effects of high cooling to subzero temperatures and hold times on membrane integrity. We determined that there was a subzero temperature range, that if the cells were held there and allowed to equilibrate with the extracellular solution, that would confer protection. This range validated the theoretical prediction of approximately 10°C supercooling. The cells were able to withstand greater than 3 M $[KCl]_i$ when the duration of time spent at the high subzero temperatures was limited. This demonstrates that virtual experiments

can be used to define ranges of experimental variables, such as subzero temperatures and duration of exposure, to minimize cryoinjury. Furthermore, it was demonstrated that simulations could be used to accurately predict biological outcomes. We were able to design optimal cooling conditions and develop a cryopreservation protocol without utilizing cryoprotectants. The cell recovery was comparable with the current clinical standard using DMSO.

7.2 Limitations of this study

While this study enabled us to successfully use simulations to cryopreserve TF-1 cells without DMSO, there are limitations that prevent us from making recommendations on HSCs for transplantation based on these findings. This research was conducted using a cell line as a model of primary cells, such as bone marrow, peripheral blood and cord blood stem cells. Tissue culture cells provide more consistent and repeatable results in both the measurement of osmotic parameters and the assessment of biological outcomes. However, now that the principle has been established, further studies with primary cells are required in order to make clinical recommendations.

For proof of principle, we used membrane integrity as the sole indicator for cell viability. Although, the membrane has been shown to be the primary site of cryoinjury, functional assays should be included prior to the use of these findings for clinical applications.

7.3 Implications of this thesis

The field of cryobiology has been dominated by research on the chemical properties of the freezing solution by the addition of various combinations and

concentrations of cryoprotectants. A refinement of that basic approach has been to modify the cooling rates used in cryopreservation procedures although a cooling rate of 1°C/min is used almost exclusively to cryopreserve cells. This thesis has now extended the approach to cryopreservation to include the properties of the cell and the physical conditions of the freezing environment. This approach was only possible through the use of modeling and simulations.

This thesis has created the opportunity for different design criteria to be used in the development of cryopreservation protocols. Previously, cryobiologists have focused on empirically maximizing recovery by changing the composition of the media and cooling rates. This thesis has shown that simulations can be used to determine conditions that minimize the two identified damaging factors in cryopreservation. Also, simulations reduce empirical experiments and allow for customizing protocols for different cell types based on their osmotic properties. It is now possible to use simulations to apply different protocol design criteria, such as alternatives to DMSO as a cryoprotectant.

7.4 General conclusions and recommendations

There is, in cryobiology, a definite need to understand the use of simulations in cryopreservation. As cryobiologists continue to further the field of scope to cells and tissues which require cryopreservation, there is an increased need in utilizing computer modeling to reduce experimentation. By combining the knowledge gained through simulations and empirical validations, a cryopreservation protocol can be established. From this thesis, modeling can be

used as a tool to answer general questions about cryopreservation protocol design, such as whether a cell can be cryopreserved without a cryoprotectant.

Simulations can be used to theoretically describe osmotic and low temperature responses of cells. Furthermore, simulations can be used to develop a cryopreservation protocol specific for a cell type, which could minimize the amount of empirical work necessary to preserve a cell type successfully. This thesis has described using thermodynamics to reduce the use of cryoprotectants, by optimizing the cooling and warming rate based on the cell type, rather than the cryoprotectant. This may have direct implications for the cryopreservation of tissues, which have multiple cell types in a matrix. It may be possible by using simulations to use the properties of the matrix as well to yield insights into cell behaviour during the freezing of a tissue. This may also prove particularly beneficial in transfusion medicine, when minimizing the amount of manipulation to the sample may reduce potential risks to the recipients. More specifically, in the case of HSC transplants, eliminating DMSO as a cryoprotectant would be very beneficial, especially for patients requiring large volumes of bone marrow or leukapheresis products.

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