Ice recrystallization inhibition as a mechanism for reducing cryopreservation injury in a hematopoietic stem cell model

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This thesis is submitted as a partial fulfillment of the M.Sc. program in Biochemistry, Microbiology and Immunology.

May 25th, 2011
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Abstract

Cryopresevation is the process of cooling biological materials to low sub-zero temperatures for storage purposes. Numerous medical and technical applications, such as hematopoeitic stem cell transplantation and sperm banking, sometimes require the use of cryopreserved cells; however, cryopreservation can induce cell injury and reduce the yields of viable functional cells. Ice recrystallization is a mechanism of cryopreservation injury, but is rarely addressd in strategies to optimize cell cryopreservation. The results from this thesis demonstrate an association between the potency of carbohydrate-mediated ice recrystallization inhibition used in the cryopreservation of umbilical cord blood and recovery of viable non-apoptotic cells and hematopoietic progenitor function. Furthermore, increased numbers of apoptotic cells in hematopoeitic stem cell grafts were associated with reduced hematopoietic function and delayed hematopoietic recovery in patients undergoing blood stem cell transplantation. These findings provide a basis for pursuing further studies assessing ice recrystallization inhibition as a strategy for improving cell cryopreservation.

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7AAD	7 actinomycin-D
ALDH	aldehyde dehydrogenase
BFU-E	Burst forming unit - erythroid
CD	cluster of differentiation marker
CFU	colony forming unit
CFU-GM	colony forming unit – granulocyte monocyte
DEAB	diethylaminobenzaldehyde
DMSO	Dimethyl sulfoxide
GVHD	graft versus host disease
HLA	Human leukocyte antigen
HPCs	hematopoietic progenitor cells
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell translpant
IRI	ice recrystallization inhibition
MGS	mean grain size
MNCs	mononuclear cells
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell
ROS	Reactive oxygen species
UCB	umbilical cord blood

Acknowledgements

The completion of this thesis required the assistance of various people to whom I am greatly thankful for their generosity and assistance.

Primarily I wish to thank my supervisor Dr. David S. Allan and my co-supervisor Dr. Robert Ben for their commitment and dedication to assisting me in completing my thesis along with their steadfast support. In particular, I would like to thank Dr. David Allan for acting as a mentor, teacher, role model and a friend throughout the project. His insight, encouragement and guidance were invaluable to my progression and completion of this thesis. In addition, I would also like to specifically thank Dr. Lionel Filion for providing me substantial support, guidance, feedback and wisdom in both the lab and in life. I would also like to thank Dr. Lisheng Wang for his feedback throughout the development of my thesis.

I am also very grateful to have worked alongside Yuhua Li, Laura Labonte and Jessie Sun who were enthusiastic, supportive and helpful colleagues throughout the completion of this project

Finally, I would like to thank MITACS-ACCELERATE Canada for the financial support they have provided to me throughout this work.

Chapter 1 Introduction

1.1 Cryopreservation methods and associated cellular injury

1.1.1 Methods of freezing

Cryopreservation is a technique commonly used to prolong the function and viability of numerous cells and tissues by storing them at subfreezing temperatures below -80^{o}C, or more commonly below -140 ^{o}C, to prevent growth of ice crystals and to slow cell metabolism to the point that cells do not age (1). Most cryopreservation methods use liquid nitrogen to maintain cells and tissues at temperatures below -196 ^{o}C (boiling point of nitrogen). Indeed,moderate recovery of viable and functional cells following cryopreservation in liquid nitrogen has been achieved with numerous cell types (2-4).

Although most cryopreservation methods involve storing cells at temperatures below -140^{o}C, the rates that cells are frozen and thawed at are not always the same. Currently, slow rate-controlled freezing and vitrification are the most widely used strategies to cool cells to temperatures below -140 ^{o}C. Slow rate-controlled freezing is the more traditional method, and typically requires freezing cells at a controlled rate of around 1 ^{o}C / minute. Vitrification, in contrast, is the method of cooling cells at very rapid rates of over 20,000 ^{o}C / minute (4). Freezing cells at slower rates of around 1 ^{o}C / minute allows for ice to form predominantly in the extracellular compartment. This is primarily due to the intracellular space being more gelatinous compared to the

extracellular space, and so the intracellular space experiences a greater colligative freezing point depression.

As ice forms in the extracellular compartment, solutes are excluded from the ice, which leads to the development of an osmotic pressure gradient across the cellular membrane. As a result, water will diffuse from the intracellular to the extracellular compartment, promoting further external ice crystal formation. This process will continue to cycle, causing the intracellular compartment to become increasingly dehydrated, until the temperature drops below a point called the glass transition temperature, where the remaining solution solidifies (5). Freezing cells at slow rates is beneficial to cell survival because it provides sufficient time for the majority of water to diffuse from the intracellular to extracellular space during freezing, and so reduces the formation of intracellular ice crystals that can be lethal to the cell (6, 7). In addition, freezing cells at slow controlled rates is also beneficial because it allows for the cell to accommodate changes in osmotic pressure without damaging the cell membrane. The freezing rate becomes particularly important throughout a phase of the freezing process, called the thermotropic phase transition, where the plasma membrane undergoes a transition from a gel to a liquid-crystalline state (8).

Despite these advantages of slow rate-controlled freezing, there are associated risks. Exposure of cells to excessive dehydration during freezing can cause the cellular membrane to rupture, which can allow for the formation of lethal ice crystals in the intracellular compartment (5). Excessive cell shrinkage from dehydration is thought to

be a potential mechanism through which membrane damage can occur (9). Dehydration also leads to increased concentrations of intracellular electrolytes, which is another predominating mechanism of cell injury during slow freezing. Injury to cells through increased electrolyte concentrations is commonly reffered to as the “solution effect”.

Another form of cellular damage that can occur during freezing is mechanical stress to cellular membranes from the formation of extracellular ice, commonly reffered to as the mechanical effect (10, 11) . A study by Takamatsu & Zawlodska(10) reported that mechanical effects contribute to cryopreservation injury throughout high subzero freezing temperatures (around -10 ^{o}C), but solution effects contribute to the majority of cryopreservation injury to cells at lower subzero freezing temperatures (below -20 ^{o}C). Increasing the freezing rate is one potential method to reduce damage from these solution effects, since freezing at fast rates will limit the time that water can be transported from the intracellular to extracellular compartments, and so cells will be less dehydrated since they will retain more water. However, increased intracellular water content also promotes the formation of intracellular ice crystals, which are extensively more lethal to cells compared to extracellular ice crystals due to their ability to damage intracellular organelles (6, 7).

Freezing cells at excessively slow or fast rates will result in extensive cellular injury (5), and so cells are typically frozen at intermediate rates of approximately 1 ^{o}C / minute to minimize cryopreservation injury. The optimal rate for freezing, however, is cell type dependent, and is largely influenced by the cell membrane permeability (9).

Erythrocytes, for example, have very permeable membranes and optimally freeze at a fast rate of -1000 ^{o}C/minute, while hematopoietic stem cells and liver cells with relatively non-permeable membranes optimally freeze at speeds approximately 1000x slower (2, 9). Freezing at these rates is typically achieved with rate controlled freezing methods, including rate controlled freezers and storage in closed containers filled with isopropyl alcohol.

1.1.2 Methods of thawing

In addition to cryopreservation-associated injury that can occur from the freezing process, cellular injury can also occur during cold storage and during the thawing process due to dynamic ice shaping and the growth of large ice crystals at the expense of small ice crystals. This phenomenon is termed ice recrystallization, and leads to an increase in the mean ice crystal size which can cause mechanical damage to cell membranes (6, 12, 13). In contrast to the freezing process, cryopreserved cells are commonly thawed in a rapid manner (37 ^{o}C water bath) to minimize cellular injury caused by ice recrystallization.

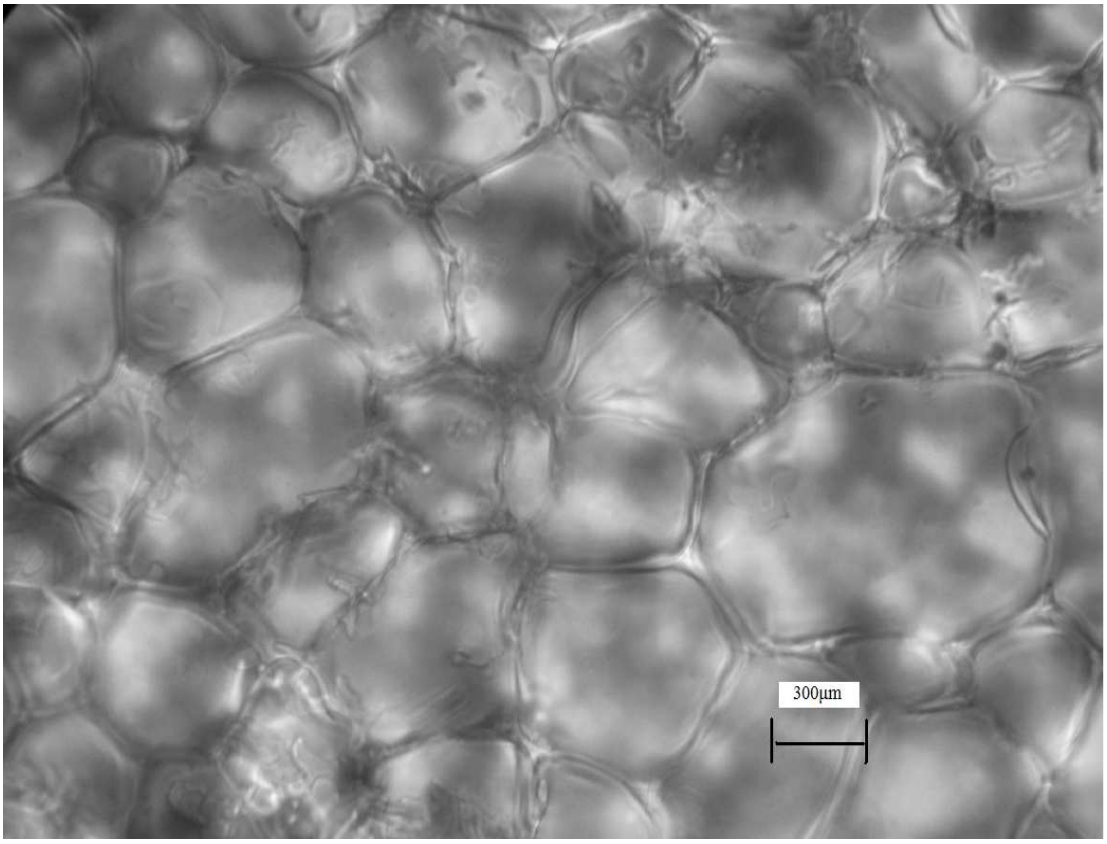
Slow thawing of cryopreserved cells has been shown to exacerbate ice recrystallization which in turn reduces cell viability (14, 15). Moreover, improved recovery of functional human β pancreatic cells following cryopreservation and rapid thawing has been demonstrated when ice recrystallization was inhibited with an analogue of antifreeze glycoproteins, an inhibitor of ice recrystallization, compared to controls (15).

Ice recrystallization associated with specific solutions can be measured with a technique originally described by Knight et al, called the splat cooling assay (16). In brief, a 10 µL droplet of a solution is released 2 m above an aluminum plate that has been cooled to -80 ^{o}C. Upon contact with the cooled aluminum plate, the droplet freezes into the shape of wafer which is composed of very fine ice grains. The wafer is then carefully removed from the aluminum plate and transferred to a cryostage dish held at approximately -6.4 ^{o}C for annealing, where the ice disc will thaw slowly.

The cryostage is then placed under a microscrope equipped with a camera and crossed polaroids to record images of the ice crytsal sizes in the ice disc as it thaws. A typical image obtained using a phosphate buffered saline (PBS) solution is illustrated in figure 1. In addition to the original technique described by Knight et al., a domain recognition software has recently been developed to determine the average size of ice crystals in the images recorded (17). The extent of ice recrystalization that occurs in solution is then represented by the average ice crystal size, or mean grain size. Larger ice crystals signifiy greater ice recrystallization.

Depending on the nature of the solution and the solutes present, the extent of ice recrystallization will differ. For instance, Tam et al. demonstrated that the extent of ice recrystallization differed in PBS solutions depending on the concentration of sugars present, in addition to the chemical structure of the carbohydrate present in solution (18). Despite the potential importance of ice

Figure 1 Typical image from the splat-cooling assay using phosphate buffered saline solution. Ice crystals are demarcated by the dark edges. The image was obtained at 76x magnification


300µm

recrystallization as a mechanism of cellular injury, many studies seeking to optimize cryopreservation of specific cell types do not address the issue of ice recrystallization.

The accumulation of cryopreservation-associated cellular injuries have been reported to affect primarily the plasma membrane (19) and cellular organelles, particularly the mitcohondria (20). Through these various cryopreservation-associated injuries, cell death can occur. Three predominant modes of cell death have been reported to occur following cryopresevation. The first is cell rupture resulting from intracellular ice formation or large fluctuations in cellular volume, as described earlier.

The second mode is cell necrosis, which is characterized by cell swelling, loss of membrane integrity, DNA fragmentation, and cytokine release. Mechanisms responsible for the third mode, cold-induced apoptosis, are not entirely clear. However, cold-induced apoptosis has been reported to be assoicated with changes in the mitochondrial membrane potential, which may activate the caspase dependent apoptotic signalling cascade through release of cytochrome C, along with the production of reactive oxygen species (ROS) that can damage intracellular structures (21, 22) Indeed, both oxidative stress from reactive oxygen species (ROS) and osmotic stressors (eg. dehydration) have been found to induce apoptotic changes in various cell types (23-25).

1.2 Cryoadditives as a strategy for reducing cryopreservation injury

To mitigate cryopreservation-associated cellular injury and cryopreservation-associated cell death, cryoprotectants are added to the cellular suspension before freezing. There are two major groups of cryoprotectants, penetrating cryoprotectants that can pass through the cell membrane unaided, and nonpenetrating cryoprotectants that cannot. Penetrating cryoprotectants generally consist of small amphipathic molecules like dimethylsulfoxide (DMSO) and glycerol, while nonpenetrating cryoprotectants typically consist of large molecules like the polysaccahride hydroxy ethyl starch and proteins. In terms of slow freezing damage, penetrating cryoprotectants such as DMSO are thought to provide the greatest cryoprotective effects by replacing water, which prevents the cell from excessive shrinking and minimizes high electrolyte concentrations in the intracellular compartments(26, 27) . In addition, DMSO also provides cryoprotective effects by reducing the ice formation at any temperature and solvates the plasma membrane (28). However, addition of DMSO at high concentrations to cellular suspensions can be toxic to unfrozen cells and may be harmful to patients if infused along with stored cells. For instance, several concerns have emerged in association with the use of DMSO for the storage of hematopoietic stem cells (29, 30). Toxicity related to DMSO infusion has been reported (31-33) and there are increasing concerns regarding the induction of apoptosis in hematopoietic stem cell grafts that are cryopreserved in DMSO (34, 35).

Antifreeze glycoproteins are proteins containing numerous disaccharide moieties with ice recrystallization inhibition (IRI) properties and confer protective effects to

organisms like polar fish when subjected to freezing and sequential thawing conditions (36). Use of antifreeze glycoproteins as cryoprotectants, therefore, has been investigated. Extracting antifreeze glycoproteins from natural sources is resource intensive, therefore, synthesis of antifreeze glycoproteins has emerged as an attractive alternative. Design of synthetic analogues also allows one to enhance the cryoprotective properties and minimize the cytotoxic properties inherent in natural antifreeze glycoproteins. Indeed, Tachibana et al. (37) demonstrated that their synthetic antifreeze glycoprotein analogues were as active as natural antifreeze glycoproteins and have significant antifreeze activity. Matsumoto et al. also demonstrated that their synthetic antifreeze glycoprotein analogues, when in combination with 10%DMSO, were able to reduce cryopreservation-associated damage and improve functionality of cryopreserved β-pancreatic islet cells compared to cryopreservation in 10% DMSO alone (15). For the continued development and optimization of synthetic antifreeze glycoprotein analogues, more insight regarding the specific properties of antifreeze glycoproteins responsible for their cryoprotective effects is needed.

Carbohydrates have also been explored as cryoprotectants, based on their protective role in specific frog species that can withstand sub-zero temperatures (38). Furthermore, the addition of carbohydrates, along with 5% DMSO, has provided encouraging results in the cryopreservation of umbilical cord blood (UCB) (39). The mechanism by which carbohydrates exert their beneficial effect has been unclear. It has been shown, however, that carbohydrates possess a natural capacity to inhibit the process of ice recrystallization via a process termed ice recrystallization inhibition (IRI)

(18), and the extent of IRI is associated with specific structural properties of the carbohydrate, including the hydration status (18, 40). Several studies have reported on the use of carbohydrates to improve UCB cryopreservation (41-43), sperm banking (3), oocyte storage (44), and human hepatocyte cryopreservation (45), but thorough structure-function studies addressing carbohydrate structure and impact on cell viability has not been performed. Furthermore, the mechanism by which the recovery of viable cells is enhanced has not been eluicdated. Greater insight regarding the role of IRI as a mechanism of carbohydrate-mediated cellular cryopreservation will allow the strategic development of novel carbohydrates and carbohyrdate based compounds with enhanced cryoprotective properties. Investigating IRI as a mechanism of carbohyrdate-mediated cellular cryopreservation is addressed in this thesis.

1.3 Applications of cryopreservation

Cryopreservation of cells and tissues has numerous applications in the fields of reproductive technologies, organ transplantation and cell-based medicine. Indeed, extensive research has been conducted over the past few decades to effectively cryopreserve germline cells (sperm and oocytes) from various species. Such applications allow for reproduction of those species in the future, and hence act as a strategy for the conservation of species, especially those that are endangered (46). Cryopreservation of human sperm and oocytes, along with ovarian tissue and embryos, is also important for the application of assisted reproduction techonolgies.

Cryopreservation of ovarian tissue, for instance, may allow women to preserve their reproductive functions for extended periods and after cancer treatments like chemotherapy that can threaten reproductive potential (47). Cryopreservation of human embryos is also crucial for *in vitro* fertilization, which is commonly used as an alternative treatment for infertility (48). Despite these various applications, numerous reports have demonstrated significant cryopreservation injury, particularly through associated apoptosis, in cryopreserved sperm (49) and ovarian tissue (50), which can result in impaired functionality.

In addition to reproductive technologies, cryopreservation is widely used for the longterm storage of liver cells and various stem and progenitors cells for cell based treatments. Liver cell transplantation can be used to treat a variety of inborn diseases in liver metabolism by supplying functional liver cells, called hepatocytes. However, cryopreservation injury to hepatocytes markedly reduces cell functionality and viability in comparison to fresh hepatocytes, and hence can affect the success of the transplantation (51).

Cryopreservation of various stem cells types, including embryonic stem cells, mesechymal stem cells and hematopoietic stem cells is also widely utilized for the longterm storage of these stem cell types for extended use in medical treatments and for research in the laboratory setting. Research on optimizing the cryopreservation of human embryonic stem cells has been an area of particular interest, since these cells provide a very efficient system to model various disease states, study human

development, and may be used as part of medical therapies in the future (52). However, human embryonic stem cells are very susceptible to cryopreservation injury, especially through cold-induced apoptotic pathways (53). Mesenchymal and hematopoietic stem cells are other types of stem cells that are stored by cryopreservation and used for medical and research based applications. Similar to embryonic stem cells, these stem cell types are susceptible to cryopreservation injury, particularly through cold-induced apoptosis (54, 55). Umbilical cord blood, an important source of hematopoietic stem cells that is routinely cryopreserved for later use, has been reported to have between 20% -70% of the stem cell population undergoing apoptosis after thawing cryopreserved units (56, 57).

Improved understanding of the mechanisms of cryopreservation injury responsible for cell death, especially through cold-induced apoptosis, will allow for the development of strategies to reduce cryopreservation-associated cellular injury and improve the utility of these stored cellular products. This thesis will address the role of IRI as a potential strategy to reduce cryopreservation-associated cellular injury and the associated loss of viable and functional cells. UCB was selected as a model system to study IRI due to the significant levels of apoptosis observed following standard UCB cryopreservation and the presence of functional relevance and importance of hematopoietic stem cells in clinical applications, including hematopoietic stem cell transplantation.

1.4 Hematopoietic stem cells and associated medical applications

1.4.1 The role of the hematopoietic system:

The haematopoietic system is an essential physiological system responsible for producing and maintaining all cellular components in the blood, including all of the mature blood and immune cells. In mammals, there is a diverse set of cells produced from the hematopoietic system, the majority of which are bone marrow derived and can be classified as either myeloid or lymphoid. Myeloid cells include progenitors and differentiated cells of the erythroid, megakaryocytic, monocytic, granuloyctic lineages, while lymphocytes and natural killer cells arise from lymphoid tissues (58). Lymphoid cells are predominantly involved in adaptive immunity, but also contribute to innate immunity, as is the case for natural killer cells. Myeloid cells, in contrast, are more diverse in function. Erythrocytes, for example, are the most common cellular component in the blood and are responsible for delivering oxygen to body tissues via blood flow through the circulatory system. Erythrocytes originate from the bone marrow and typically circulate throughout the circulatory system for approximately 100-120 days before being recycled by macrophages. Granulocytes include neutrophils, eosinophils and basophils. The most abundant type of granulocytes are neutrophils and exert numerous antimicrobial functions, including; phagocytosis, degranulation, neutrophil extracellular traps, and release of cytokines to amplify inflammation reactions. As a result, neutrophils are an essential component in the innate immune system. The life span of neutrophils, however, is limited to approximately 5 days when inactivated, and reduced to about 2 days when activated (59). The role of neutrophils as an essential front line defense against invading pathogens, coupled with limited life span,

necessitates extensive and continuous production. Platelets arise from megakaryocytes in the bone marrow and are central in the process of blood coagulation and hemostasis. Platelets have a limited lifespan of approximately 5-9 days. These important myeloid blood cell types, together with lymphoid cells, demonstrate the diverse functions of the hematopoietic system and its critical role in maintaining life. Maintenance of the hematopoietic system depends on the constant maturation of functional blood cells from immature stem and progenitor cells.

1.4.2 Hematopoiesis: the role of stem and progenitor cells

Following numerous experiments in the early 1960's on the transplantation of limited bone marrow cells into mice, Till and McCullough identified a rare specialized group of blood cells, termed hematopoietic stem cells (HSCs), that possessed both the ability to regenerate themselves and produce various types of myeloid and erythroid cells *in vitro* (60-62). Later studies in the early 1990's confirmed these same capacities of hematopoietic stem cells *in vivo*, by demonstrating that a single HSC could generate all of the mature blood cell types in a recipient mouse for over 6 months, and could continue to do so upon secondary transplantation into another mouse (63-65). These capacities to self-regenerate and produce multiple blood cell types came to be later defined as self-renewal and multipotency, respectively. (66). It is through both self-renewal and differentiation of hematopoietic stem cells (HSCs) into all of the various mature blood cell types that the hematopoietic system is able to produce and maintain a sufficient supply of blood and immune cells throughout an organism's life span (65).

During normal physiological states, a rapid rate of over 1 million mature blood cells are being produced per second (67). This extensive quantity of mature blood cell production would seem to necessitate an extensive and rapid rate of HSC proliferation and differentiation. Interestingly, however, the majority of hematopoietic stem cells residing in the adult animal bone marrow have been shown to be at rest in the G_0 stage, cycling very rarely (68, 69). Instead, extensive proliferation and differentiation of mature blood cell precursors derived from HSCs, termed hematopoietic progenitor cells (HPCs), is thought to be the predominant mechanism by which the hematopoietic system is able to turn over such vast quantities of new mature blood cells (70).

These hematopoietic progenitor cells (HPCs) differ from HSCs in 2 ways: 1) HPCs have lost the capacity for indefinite self-renewal that HSCs possess and 2) HPCs are generally more restricted compared to HSCs in their potency for differentiation into the various mature blood cell types. In addition, HPCs are a heterogeneous population, ranging from oligopotent progenitors that possess the ability to differentiate into several mature blood cell types to unipotent progenitors that are restricted to differentiating into only one type of mature blood cell.

Hematopoiesis is hierarchical, with the least lineage-restricted progenitors deriving directly from HSCs to more committed progenitors and mature cells through stages of lineage-restricted differentiation. Moreover, two pools of hematopoietic stem cells have been characterized, termed long-term and short term repopulating HSCs (71-73) with different capacities for self-renewal. Long term HSCs possess life-long self-

renewal while short term HSC have limited self-renewal capacity. Common myeloid progenitors and common lymphoid progenitors, however, are more committed precursors that give rise to their respective cell lineages. Indeed, it has been shown that common myeloid progenitors give rise to all myeloid cells, but are unlikely to give rise to B lymphocytes (74). Likewise common lymphoid progenitors have been shown to possess the capacity to differentiate into all lymphoid cells, but typically do not differentiate into myeloid cells under normal physiological conditions (75). Certain aspects of this classical model of hematopoiesis, however, have been challenged, more specifically with regards to the lineage restriction of the common myeloid and lymphoid progenitors. For instance, Kawamoto *et al.* recently demonstrated that early progenitors in the thymus retain myeloid potential. To account for this, they proposed a slightly different model called the myeloid-based model, which posits that myeloid cell differentiation potential is maintained in B and T lymphocyte precursors, even after these lineages segregate from each other (76).

HSC and HPC populations differentiate into more mature progenitors or blood cells, the phenotypic expression of many cell surface markers changes. As such, the set of cell surface markers present on a cell can act as a surrogate marker for the stage of cellular differentiation, self-renewal capacity and functionality. One of the most widely used cell surface markers to distinguish HSCs and HPCs from mature blood cells is a cell surface marker called CD34, which is a single-pass transmembrane protein of the sialomucin family that is expressed predominantly in early hematopoietic and vascular tissue, including HSCs and HPCs (77) . Although the exact function of CD34 has not

been definitively determined, CD34 has been shown to be implicated in promoting HSC and HPC self-renweal, while inhibiting those populations from differentiating, enhancing HSC and HPC homing and trafficking to bone marrow for maintenance, and both promoting and blocking cell-cell adhesion (77). Evidently, CD34 is likely an essential protein for proper functioning of HSCs and HPCs, making it an appropriate surrogate for HSC and HPC identification. Indeed, CD34 was the first marker to be used for isolation of HSCs and almost all $CD34^+$ cell populations have been shown to possess multipotency or oligopotency (78-80). Another important surface marker that has been used to discriminate between HSC/HPCs and mature blood cells is the protein tyrosine phosphatase receptor type c, more commonly referred to as CD45 (81, 82). It is a transmembrane protein, similar to CD34, but unlike CD34, CD45 is expressed specifically on all differentiated hematopoietic cells, except erythrocytes and platelets (83). CD45 has been shown to play important roles in regulating a variety of cellular processes, including; cell differentiation and cell growth. In combination with CD34 and CD45, the absence of the cell surface marker CD38, and expression of the cell surface marker CD90, have been used to further identify earlier multipotent HSC populations more specifically (84, 85). In addition, identifying cells with high activity of an enzyme called aldehyde dehydrogenase (ALDH), in combination with all these cell surface markers, has been shown to allow for the isolation of hematopoietic stem cells at very high purities (86). This is likely because ALDH has been shown to play an important role in HSC differentiation by regulating the retinoic acid signaling pathway (87).

1.4.3 Application of hematopoietic stem and progenitor cells for medical applications

Hematopoiesis, although essential to the maintenance of life, can also become aberrant or unregulated and lead to leukemia and lymphoma. Leukemia typically involves the bone marrow compartment causing a marked decrease in normal hematopoieis and lymphoproliferative disorders such as lymphoma typically involve lymph nodes. In either case, patients with leukemia and lymphoma are often treated with systemic chemotherapy and/or radiation treatment. Both chemotherapy and irradiation impair cell replication and induce cell death. While eradicating the malignant cells, chemotherapy and radiation also inhibit cell replication and induce cell damage in the hematopoietic system due to the relatively high rate of cell cycling in the bone marrow. For instance, neutrophils and platelets are especially sensitive to chemotherapy and radiation. In conventional dosing of chemotherapy or irradiation, the patient's blood cells will decrease for a brief period, followed by recovery to normal levels. However, because advanced hematological malignancies may respond favorably to increasing doses of chemotherapy or irradiation, some patients will undergo dose escalations that can be curative but eliminate the bone marrow as a consequence, a treatment called myeloablation. Recovery of mature blood cells after myeloablative treatment requires the transplantation of hematopoietic stem cells, a procedure termed hematopoietic stem cell transplantation (HSCT), to avoid prolonged neutropenia (low neutrophil levels in the blood), and the associated risk of infection from microbial pathogens and to avoid thrombocytopenia (reduced quantities of platelets in the blood), that is associated with bleeding complications.

Hematopoietic stem cell transplantation (HSCT) following myeloablation is essential for recovery of mature blood cells, especially fast growing cells like neutrophils and platelets. Minimizing the period of insufficient blood cell production, termed cytopenia, following myeloablative treatment reduces the risk of infection and excessive bleeding, and contributes to the success of safe and effective transplantation. The time between HSC transplantation and recovery of sufficient blood cells is termed engraftment. In a patient setting, the exact definition of engraftment can vary, but numerous studies have defined engraftment as the number of days required to reach a concentration of $> 0.5x10^9$ neutrophils/L blood and $> 20x10^9$ platelets / L blood for three consecutive days (88-90). The rapid reconstitution rates and critical roles that neutrophils and platelets have in immunity and wound healing, respectively, may explain why these cells have been chosen as markers of engraftment. However, recent studies have reported on other cell types like reticuloytes as earlier markers of hematopoietic engraftment (91).

Several sources of cells can be used for HSCT, including bone marrow, mobilized peripheral blood or UCB. In addition, each of these stem cell sources can be collected either from the patient themselves, termed an autologous transplant, or from another person, termed an allogeneic transplant. Autologous HSCT is most commonly performed to allow patients to undergo higher doses of chemotherapy and radiation in an effort to eradicate remaining cancer cells. Typical diseases that can be better controlled using higher doses of treatment that require hematopoietic rescue with autologous HSCs include relapsed or high-grade lymphomas and multiple myeloma, a

cancer of plasma cells. The process of autologous HSCT requires the collection of the patient's own stem cells, followed by cryopreservation of those stem cells, and then reinfusion of the stem cells at a later date following the completion of myeloablative chemo/radiotherapy.

In allogeneic stem cell transplantation, HSCs are collected from human leukocyte antigen (HLA) compatible sibling or unrelated donor, including UCB. Allogeneic transplantsgenerally require strict matching between the recipient and donor for 6 or more genes of the human leukocyte antigen (HLA) cluster. When the recipient of an allogeneic HSC transplant is not sufficiently matched with the donors HLA alleles, there will be an increased risk for two major immune-mediated complications, graft rejection and graft versus host disease. In graft rejection, the HSC graft is rejected by the recipients own immune system, while in graft versus host disease the immune cells within the graft initiate an immune response against the recipient's own tissues. These immunological rejection events, along with transplantation of insufficient cell doses can result in failed engraftment.

Despite the increased risk of immune-related complications, allogeneic transplantation can be advantageous since it also provides a platform for beneficial immunological reactions against the tumor, termed graft versus leukemia, where mature immune cells in the graft initiate an immune response against cancer cells. The need for strict HLA matching is less stringent when UCB is used as the source of stem cells, and so UCB has become an attractive alternative source of HSCs for allogeneic stem

cell transplantation when a matched sibling or unrelated donor cannot be identified. Although enriched for HSCs, UCB units are limited by the total volume available, which can influence the total dose of stem cells and contribute to delayed engraftment. Moreover, as with autologous stem cell grafts, UCB units require storage until use, most commonly by cryopreservation in liquid nitrogen. Cells collected from sibling or unrelated allogeneic donors, however, do not require cryopreservation but instead are collected just prior to their use and typically are infused “fresh”. As previously mentioned, subjecting UCB grafts to cryopreservation can injure stem and progenitor cells, further exacerbating the problem of a limited dose of viable functional cells that can engraft after transplantation. Particular issues associated with cryopreservation of autologous cells and UCB remain a challenge in HSCT and may limit more widespread use of UCB in particular.

Peripheral blood stem cell (PBSC) grafts have become the preferred source of autologous HSCs due to ease of collection and more rapid engraftment rates compared with bone marrow following meyloablative chemotherapy and/or radiation treatment. (92, 93) The most important criteria for predicting engraftment rates following PBSC transplantation has been the dose of hematopoietic stem and progenitor cells infused. More specifically, hematopoietic populations possessing the cell surface marker CD34 correlate with timely engraftment. Indeed, CD34 is a surrogate marker of oligopotent and multipotent hematopoietic populations. Numerous studies have demonstrated the quantity of $CD34^+$ progenitors within a PBSC graft to be the best predictor for the rate of engraftment, as determined by neutrophil and platelet recovery (94-97). Other factors

including the intensity of chemotherapy and/or radiation, age and sex of the recipient have also been shown to influence neutrophil and platelet engraftment (94, 98, 99). Due to the importance of the $CD34^+$ cell dose infused, multiple studies have identified minimum threshold quantities of $CD34^+$ progenitors that should be infused to ensure rapid engraftment. Typically, infusion of 2.0 x 10^6 $CD34^+$ progenitors/kg has been demonstrated to be the minimum threshold associated with prompt engraftment following PBSC transplantation (100, 101). However, the CD34 content of a PBSC graft alone does not always predict rapid engraftment. In autologous peripheral blood stem cell transplants, the number of viable $CD34^+$ cells following cryopreservation has been shown to be markedly decreased due to the adverse effects of processing and storing the cells and this reduction in the CD34+ cell dose has been shown to prolong engraftment rates (102, 103).

Although most patients engraft in a rapid manner when transplanted with PBSC grafts possessing greater than 2.0 x 10^6 $CD34^+$ progenitors/kg, there are still a small number of patients with delayed recovery of blood counts. The percentage of patients that experience delayed engraftment despite receiving sufficient $CD34^+$ progenitors varies. A study by Trébéden-Negre *et al.*(104) found 16 out of 246 patients developed delayed neutrophil engraftment after receiving at least 3 x10^6 $CD34^+$ progenitors/kg. These 16 patients did not have any known clinical factors that were likely to adversely affect engraftment. Similar findings are further demonstrated in a set of case studies described by Mineishi *et al.*(105), in which 4 patients receiving a sufficient dose of $CD34^+$ progenitors (>4.0x10^6 $CD34^+$ progenitors / kg) were identified yet experienced

delayed neutrophil engraftment. It is possible that some of the cells included in their counts were apoptotic yet were still enumerated as viable $CD34^+$ cells. Apoptotic cells could have reduced engrafting potential and contribute to delayed hematopoietic recovery after transplantation.The impact of cold induced apoptosis on engrafting potential of UCB and PBSC requires further study and was addressed in this thesis as a model system of cryopreserved cell storage.

1.5 Statement of the problem

Cryopreservation can injure cells and reduce the yield of viable functional cells. Cold-induced apoptosis has been reported to be a predominant form of cell death in many cryopreserved cells and may reduce the functionality of stored cellular products. Cold-induced apoptosis is particularly relevant for UCB banking where cells are often cryopreserved for extended periods of time, and the levels of cold-induced apoptosis become more prevalent as the duration of cryopreservation increases. Ice recrystallization is a process that occurs during thawing of cryopreserved solutions and is characterized by growth of large ice crystals, which can cause mechanical damage and apoptotis to cells in the cryopreservation solution. Ice recrystallization, therefore, could be a dominant contributor to cold-induced apoptosis and reduced functionality of cryopreserved cells and requires further study.

1.6 Objectives

This thesis will address carbohydrate mediated ice recrystallization inhibition as a possible mechanism to reduce cryopreservation injury and loss of cell functionality that occurs from ice recrystallization during the cryopreservation and thawing process. Our studies will focus on the cryopreservation of human UCB. The thesis also aims to determine if increased cold-induced apoptosis in stored HSC products is associated with reduced hematopoietic function. These studies will provide a platform for future research that endeavours to reduce cold-induced apoptosis through inhibition of ice recrystallization.

1.7 Hypothesis

More potent inhibitors of ice recrystallization will improve the cryopreservation of UCB cells, as measured by improved recovery of non-apoptotic (Annexin-V^{-ve}) cells and greater recovery of hematopoietic function, compared to less potent inhibitors of ice recrystallization. Furthermore, the practical relevance of cold-induced apoptosis will be demonstrated by correlating the number of apoptotic cells in blood stem cell products with delayed hematopoietic recovery in patients undergoing hematopoietic stem cell transplantation.

Chapter 2: Materials and Methods

2.1 Collection, storage and processing:

2.1.1 Umbilical cord blood (UCB)

Umbilical UCB was collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of The Ottawa Hospital according to research protocol #2006460-01H (performed by collaborators at The Ottawa Hospital). UCB was decanted into 50ml falcon tubes and centrifuged (Allegra X-15R centrifuge, Beckman Coulter) at 350xg for 10 minutes to remove red blood cells. The remaining buffy coat mononuclear cells (MNCs) and plasma were recovered. MNCs were were then diluted 1:2 in the autologous plasma (15mL of buffy coat MNCs in 15mL autologous plasma) and then layered on ficoll with at 1 part (15mL) ficoll(Ficoll-Hypaque, GE Healthcare): 2 part plasma/buffy coat (30mL). Samples were then run under ficoll gradient centrifugation at 400xg for 30 minutes and then washed twice in PBS at 300xg for 10 minutes (Allegra X-15R centrifuge, Beckman Coulter). Total MNCs (1.5-2.5 x 10^7 cells/mL) were then suspended in 1 ml media RPMI containing 37% Pentaspan (v/v) (a membrane stabilizer used by Canadian blood services at similar concentrations (37% v/v) for cryopreservation of peripheral blood stem cell grafts) and carbohydrate solutions at concentrations of either 20mM, 200mM or 500mM.

A total of 2 replicates were analyzed for 5 different sugars (glucose, galactose, melibiose, trehalose and sucrose) at 20mM, 200mM and 500mM to identify an optimal

concentration for cryopreservation. An additional 3 replicates for each sugar at 200mM was also analyzed once 200mM was identified as the optimal concentration. 1ml Cell suspensions were cryopreserved in 2 mL cryogenic vials (Corning Incorporated, New York) under rate-controlled conditions to -80 ^{o}C (Mr. Freezy, Nalgene Labware) over 16h and then transferred to liquid nitrogen at least 1 week before thawing and analyzing. The duration of 1 week was chosen for logistical reasons and because this duration has been used in previous studies on UCB cryopreservation (106).One sample from each UCB unit was also stored using 5% DMSO in replacement of sugars (Sigma-Aldrich) as an internal standard for each sample. After one week of storage, samples were thawed in a 37 ^{o}C water bath (rapid thaw). An additional 2 replicates of each sugar at 200mM were subjected to the same cryopreservation conditions, but thawed at ambient temperatures (slow thaw).

Once thawed, 0.8mL of the samples were run through a filter [need microns] (Miltenyi Biotec, Germany) into 15 mL falcon tubes and diluted with 4 mL RPMI solution at 37 ^{o}C containing 50x10^{3} units/mL DNase I, (Sigma-Aldrich, Germany) and 5 mM $MgCl_2$ (Sigma-Aldrich, Germany). Samples were incubated in air for 4 minutes and then further diluted with another 5 mL of the RPMI/DNase I solution. Subsequently, samples were centrifuged for 10 min at 300 g's at 4 ^{o}C. The supernatant was then removed and the cells were resuspended in 1 mL RPMI media to prevent prolonged exposure to DMSO. Cell concentrations were then determined using a hemocytometer for further analysis by flow cytometry and colony forming unit assays.

2.1.2 Peripheral blood stem cell grafts

Patient selection was performed by Dr. Ayman Al-Hejazi, a bone marrow transplant fellow at The Ottawa Hospital in 2007. In brief, patients who underwent autologous PBSC transplantation for non-Hodgkin's lymphoma at the Ottawa hospital between January 2000 and December 2005 and who provided consent for the use of medical information for research purposes in accordance with the Ottawa Hospital's research ethics board were included in the study. Research was performed according to research protocol number 1997509-01H Cases were defined as patients with a persistent neutrophil count < 1.0×10^9/L at day 30. Patients from the same cohort that had a neutrophil count > 1.0×10^9/L prior by day 30 were selected as controls. Controls were matched for various factors, including age, sex and disease type. Other factors matched between cases and controls are outlined in table 1. All cases and controls were ≥ 18 years of age. A total of 13 delayed neutrophil engraftment cases and 22 matched controls were analyzed. PBSC grafts were volume reduced by centrifugation, transferred into an equal volume of a cryoprotectant solution containing 10%DMSO, 8% human serum albumin, 43.2% pentaspan and 14.8% Plasma-Lyte A and then placed directly into liquid nitrogen vapour overnight for cryopreservation (Performed by collaborators at Canadian Blood Services).

Cryopreserved aliquots containing approximately 1mL of a cryopreserved cellular suspension, separate from the PBSC grafts, were thawed in a 37˚C water bath until no ice could be seen and then analyzed for this study. Once thawed, 0.5mL of the sample

was transferred into a 15mL centrifuge tube and diluted 6 fold, dropwise over 5 minutes, with RPMI solution at 37˚C containing 5.0 x 10^4 units/mL DNase I, (Sigma-Aldrich, Germany) and 5 mM MgCl2 (Sigma-Aldrich, Germany). Subsequently, samples were centrifuged (Allegra X-15R centrifuge, Beckman Coulter) for 10 minutes at 250 x g at 4 ^oC. The supernatant was then removed and the cells were resuspended in 2 mL RPMI media to prevent prolonged exposure to DMSO. Cell concentrations were then determined using a hemocytometer for further analysis by flow cytometry and colony forming unit assays.

2.2 Colony forming unit assays:

2.2.2 Colony forming unit assay methodology

Clonogenic assays were performed on thawed UCB and PBSC aliquots using methylcellulose media (Methocult GF H4434; Stemcell Technologies, Vancouver, Canada) in accordance with manufacturer's instructions. Briefly, cells were suspended in 1 mL of IMDM media with 2% fetal bovine serum (Stemcell Technologies, Vancouver, Canada) at a concentration of 1 x 10^6 cells/mL for peripheral blood stem cell samples and of 5 x 10^5 cells/mL for UCB samples. A volume of 0.3 mL of the cell suspension was transferred into 2.7 mL of the methylcellulose media to obtain a final concentration of 1 x 10^5 cells/mL for peripheral blood stem cell samples and 5 x 10^4 cells/mL for UCB samples. 1.0 mL of the methylcellulose cell suspension was plated in duplicate into 35 mm diameter Petri dishes and incubated at 37 ^oC and 5% CO_2 for 14 days. Colony

forming units (CFU) were enumerated by morphology using an inverted microscope to discriminate erythroid (CFU-E), erythroblast-forming units (BFU-E), granulocyte/macrophage (CFU-GM), and granulocyte/erythroid/macrophage/ monocyte (CFU-GEMM) colonies (more than 50 cells/colony).

2.3 Flow cytometry:

2.3.1 Justification for measuring viability with 7AAD and apoptosis with Annexin-V

Numerous flow cytometric criteria are assessed when transplanting HSC grafts to ensure that the graft will transplant successfully. In HSC grafts that have been cryopreserved, measurement of $CD34^+$ cell viability has been shown by Allan et al. (102) and Lee et al.(103) to be an important criterion for predicting rates of engraftment in autologous peripheral blood stem cell grafts, while Scaradavou et al. (107) has shown $CD34^+$ cell viability to be important for predicting successful engraftment of UCB grafts. All of these studies identified viable cells by measuring the exclusion of a fluorescent DNA binding dye, called 7-actinomycin D (7AAD) via flow cytometry. The use of 7 actinomycin-D as a viability marker was first described by Schmid et al.(108) in 1992 and has become a widely accepted viability marker by numerous transplant centers. Indeed, the International Society for Hematotherapy and Graft engineering (109) recommend measurement of $CD34^+$ cell viability through flow cytometric analysis of $CD34^+$ cells excluding 7AAD, especially in stem cell grafts that have been cryopreserved.

7AAD permeates into cells that have lost their membrane integrity and are no longer viable. Since the loss of membrane integrity occurs very late in the process of cell death, 7AAD is not able to identify cells in early stages of apoptosis. Multiple studies have identified earlier markers of apoptosis, including annexin V (55, 110). Annexin-V is a protein that binds to a phospholipid called phosphatidyl serine, which is usually only externalized on the outer membrane of cells undergoing apoptosis. Externalization is typically caspase activation dependent, but can also result from caspase independent apoptotic mechanisms. Disruption of the mitochondiral membrane potential also leads to phosphatidyl serine externalization (111). Irrespective of the mechanism, phosphatidyl serine externalization occurs before the loss of cell membrane integrity in cells undergoing apoptosis (112) and measuring Annexin-V binding represents the quantity of cells undergoing early apoptosis. Furthermore, Shin *et al.* have demonstrated that early apoptotic $CD34^{+}$ progenitors, as measured by Annexin-V,(56) isolated from umbilical UCB are significantly impaired in their capacity to engraft NOD-SCID mice compared to nonapoptotic $CD34^{+}$ progenitors ($AnnexinV^{-ve}$) (56). Based on the functional relevance of Annexin-V it was selected as a marker for early apoptosis and 7AAD was selected as a marker for cell death (ie. non-viable). As a result, Annexin-V(+) 7AAD(-) cells will be defined as viable apoptotic and cells that are Annexin-V(-) 7AAD(-) will be defined as viable nonapoptotic. 7AAD(+) cells are non-viable, or dead.

2.3.2 Methodology for flow cytometry of umbilical cord blood

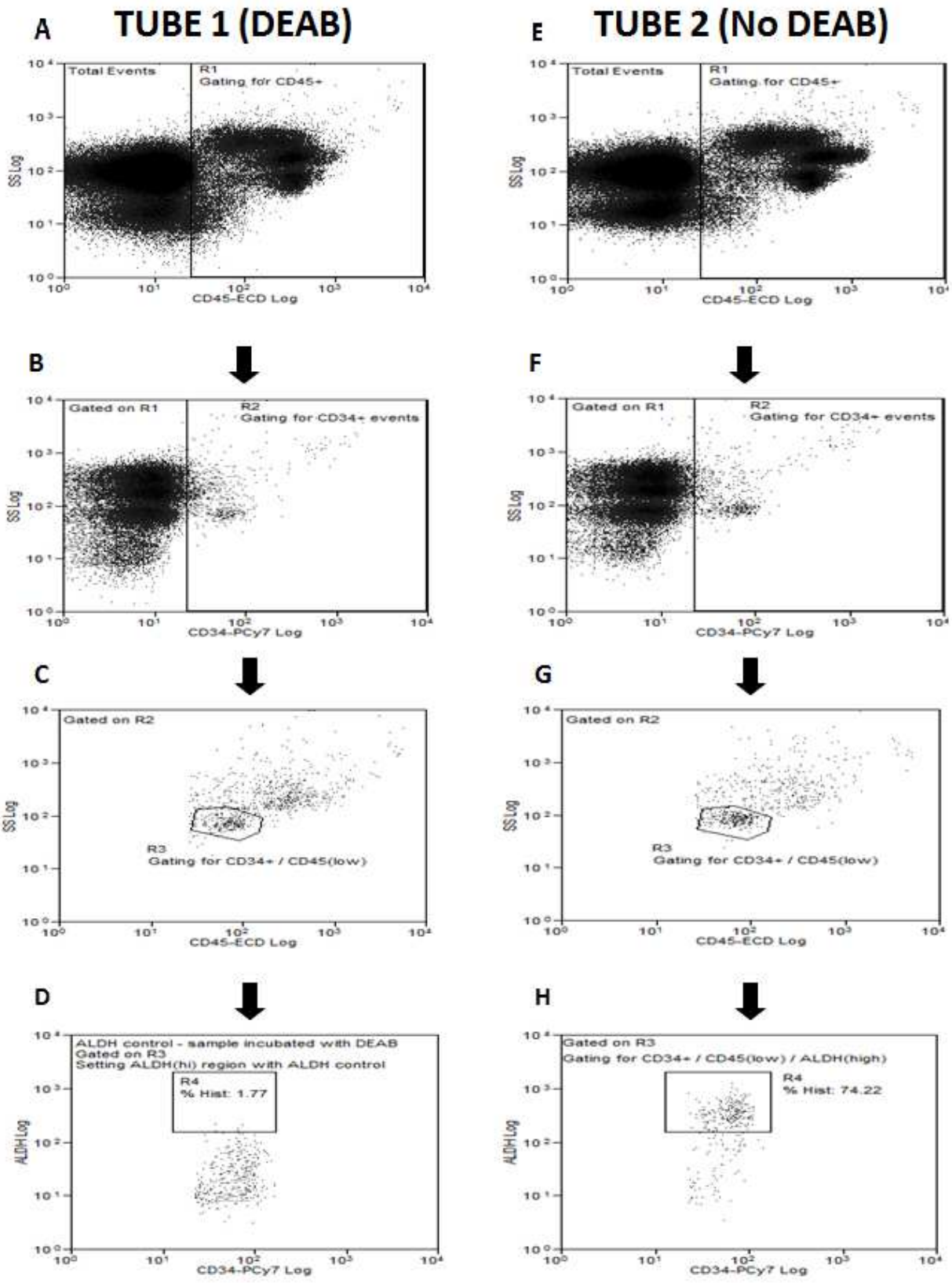
Cells were analyzed before and after cryopreservation for viability and apoptosis. MNCs ($2x10^6$ cells/ml) were suspended in Annexin-V binding buffer (BD Pharmigen, San Diego). 100 µL of the cell suspension was placed into 12x75 polystyrene tubes (Becton Dickinson) and stained with anti-CD34- fluorescein isothiocyanate (BD Pharmigen, San Diego) , Annexin V- phycoerythrin (BD Pharmigen, San Diego) , and 7AAD (BD Pharmigen), San Diego) and incubated at room temperature for 20 minutes. Samples were diluted up to 500 µL with Annexin-V binding buffer following incubation. Unstained Aliquots were used to set the control gates. Cells were immediately analyzed with a BD LSR I flow cytometer (BD Biosciences). Data analysis was performed using the software Summit (DAKO). Relative viabilities were obtained by dividing the percentage of $7AAD^-$ cells from the respective sample by the percentage of $7AAD^-$ cells from the 5% DMSO control to standardize the viabilities. Relative viable non-apoptotic cells were obtained by dividing the percentage of Annexin-V^- / $7AAD^-$ cells from the respective sample by the percentage of Annexin-V^- / $7AAD^-$ cells from the 5% DMSO control to standardize the viable nonapoptotic cells.

2.3.3 Methodology for flow cytometry of peripheral blood stem cell grafts

Cells were analysed by 5-color flow cytometry to measure viability and apoptosis in the specific cell populations shown in figure 2. Examples of histograms from these dot plots can be found in Appendix B. All fluorescent markers were incubated together in one tube. Following thawing of cryopreserved samples, cells were washed in 5 volumes

RPMI and centrifuged at 250xg for 10 minutes to remove DMSO. Cells were then resuspended in 2 mL RPMI. To identify aldehyde dehydrogenase-expressing (ALDH) progenitor cells, 4 x 10^6 cells were suspended in 1mL of Aldecount assay buffer (Stemcell Technologies, Vancouver, Canada). 0.5 mL of the cell suspension was diluted with 1.5 mL activated Biodipy–aminoacetaldehyde (BAA) (Stemcell Technologies, Vancouver) into a tube labeled tube 2. 500 µL of this original sample was immediately added to 10 µL of diethylaminobenzaldehyde (Stemcell Technologies) in a separate tube, labeled tube 1, to inhibit ALDH activity and act as a negative control. Both tube 1 and 2 were incubated at 37 ^{O}C for 30 minutes, centrifuged at 250 x g for 10 mins and resuspended in 100 µL of Annexin-V binding buffer (BD Pharmigen, Mississauga, Canada). Anti-CD34-phycoerythrin cyanin 7 (clone #581, Beckman Coulter, Mississauga, Canada), Annexin V- phycoerythrin (BD Pharmigen), 7AAD (BD Pharmigen) and CD45-electron coupled dye (clone # J.33, Beckman Coulter) were then added in combination into both tubes 1 and 2, and incubated at room temperature for 20 minutes. Both tubes were then diluted with Annexin-V binding buffer and analyzed with a FC500 flow cytometer (Beckman Coulter). Hematopoietic $CD34^+$ progenitor cells were identified using a modified version of the international standards for hematotherapy and graft engineering (113). Data analysis was performed using the Summit software (DAKO, Carpinteria, United States). The $CD34^+$ $CD45^{low}ALDH^{high}$ progenitor region was set by the fluorescence of BAA in $CD34^+$ progenitors from the control tube where the biodipy-aminoacetaldehyde catalysis was inhibited. $CD34^+$ $CD45^{low}ALDH^{high}$ progenitor cells were measured to identify early hematopoietic progenitors as compared to more differentiated $CD34^+$ $CD45^{low}ALDH^{low}$ progenitors.

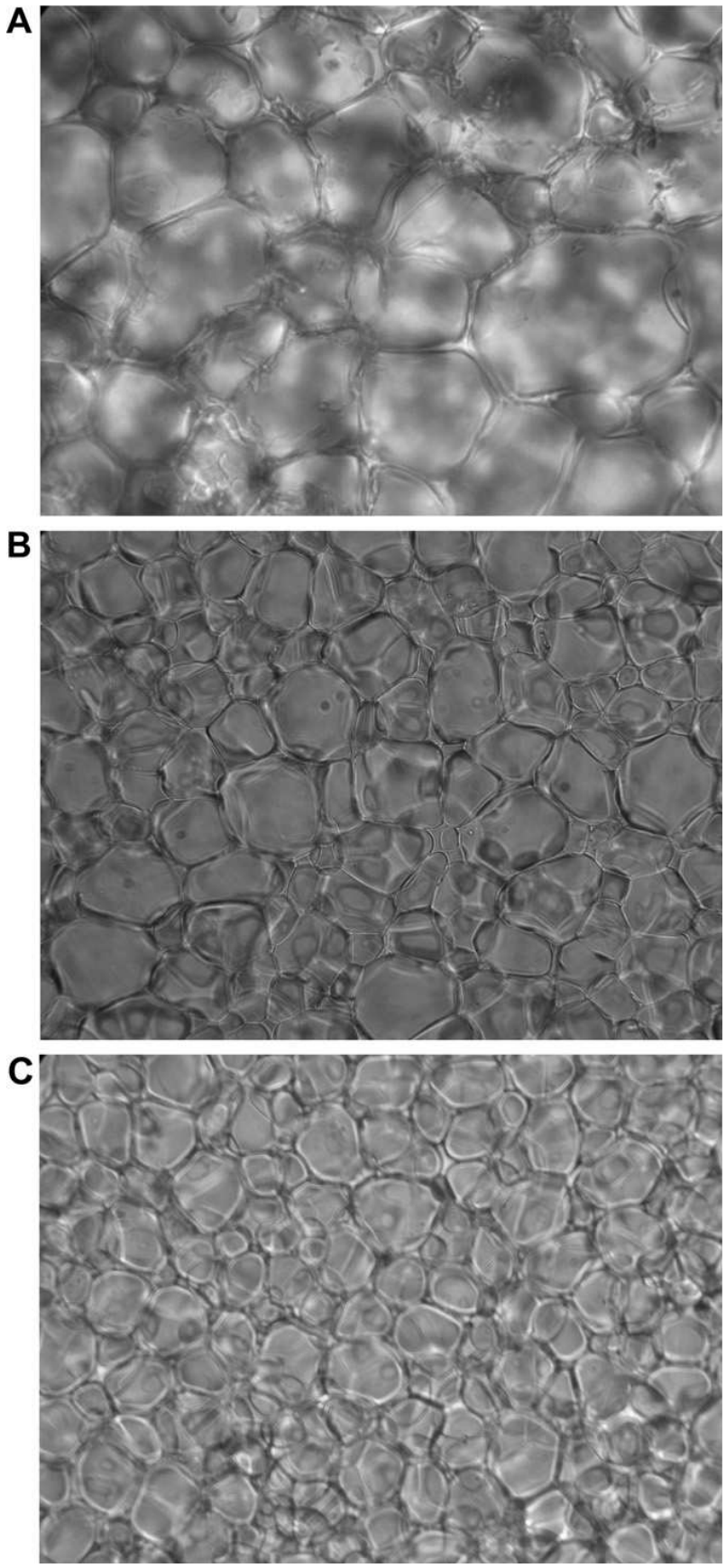
Figure 2. The gating strategy used to identify the various cell populations using flow cytometry. To identify the the $ALDH^{high}$ population, a portion of each original sample (tube 2) was incubated with diethylaminobenzaldehyde (DEAB) to inhibit ALDH catalysis of the fluorescent marker biodipyaminoacetaldehyde (tube 1). Both tubes 1 and 2 were stained with Anti-CD34- phycoerythrin cyanin 7, Annexin V- phycoerythrin, 7AAD, CD45-electron coupled dye and activated biodipyaminoacetaldehyde in combination. A modified version of the International Society for Hematotherapy and Graft Engineering gating strategy was utilized to define CD34+ progenitors. Total events are depicted in **A** and **E** and were analyzed for the $CD45^{+}$ events as defined by R1. R1 was then gated onto **B** and **F** and analyzed for $CD34^{+}$ events as defined by R2. R2 was then gated onto **C** and **G** and analyzed for $CD34^{+}CD45^{low}$ events as defined by R3. $CD34^{+}$ progenitors are defined as the $CD34+CD45^{low}$ population identified in R3 of **C** and **G**. R3 was then further gated onto **D** and **H.** The $CD34^{+}CD45^{low}ALDH^{high}$ progenitor region was defined by R4 in **D** where ALDH activity was inhibited by (DEAB) . $CD34^{+}CD45^{low}ALDH^{high}$ cells are defined in **H**. ALDH – aldehyde dehydrogenase DEAB - diethylaminobenzaldehyde


A
TUBE 1 (DEAB)
Total Events
R1
Gating for CD45+
SS Log
CD45-ECD Log
B
Gated on R1
R2
Gating for CD34+ events
SS Log
CD34-PCy7 Log
C
Gated on R2
R3
Gating for CD34+ / CD45(low)
SS Log
CD45-ECD Log
D
ALDH control - sample incubated with DEAB
Gated on R3
Setting ALDH(hi) region with ALDH control
R4
% Hist: 1.77
ALDH Log
CD34-PCy7 Log
E
TUBE 2 (No DEAB)
Total Events
R1
Gating for CD45+
SS Log
CD45-ECD Log
F
Gated on R1
R2
Gating for CD34+ events
SS Log
CD34-PCy7 Log
G
Gated on R2
R3
Gating for CD34+ / CD45(low)
SS Log
CD45-ECD Log
H
Gated on R3
Gating for CD34+ / CD45(low) / ALDH(high)
R4
% Hist: 74.22
ALDH Log
CD34-PCy7 Log

2.4 Ice recrystallization-inhibition (IRI) assay:

IRI activity of all sugars utilized in the study were determined by collaborators, Jackie Tokarew & Jennifer Chaytor, graduate students in the department of chemistry at University of Ottawa, using the splat cooling assay described earilier in section 1.1.2. A total of three images were taken from each wafer using a Nikon CoolPix 5000 equipped to the cryostage. Typical images are shown in Figure 3. During flash freezing, ice crystals spontaneously nucleated from the supercooled solution. These initial crystals are relatively homogeneous in size and quite small. During the annealing cycle, recrystallization occurs, resulting in a dramatic increase in ice crystal size. A quantitative measure of the difference in recrystallization inhibition of two compounds X and Y is the difference in the dynamics of the ice crystal size distribution, termed the mean grain size (MGS). Compounds with stronger IRI activity will result in smaller MGS. Image analysis of the ice wafers was performed using a novel domain recognition software.

Figure 3 Light microscope image of PBS solution following splat-cooling assay (**A**). Ice crystals are demarcated by the dark edges. Light microscope image of 200 mM (w/v) galactose in PBS solution following splat-cooling assay (**B**). Ice crystals are demarcated by the dark edges and appear smaller than in image 3SA. Light microscope image of 200 mM (w/v) melibiose in PBS solution following splat-cooling assay (**C**). Ice crystals appear the smallest in **3C** in comparison to **3A** and **3B**. Images shown are at 76x magnification. Ice crystal sizes were measured with a domain recognition software and used to calculate the IRI capacity of a carbohydrate by dividing the average ice crystal size in the solution containing the carbohydrate by the average ice crystal size in a solution of PBS.


A
B
C

2.5 Statistical analyses

GraphPad Prism version 5.00 (Graphpad Software, San Diego, California) for Windows was used for statistical analysis. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test with a significant value of $p < 0.05$. Data were plotted using Microsoft Excel or Graphpad.

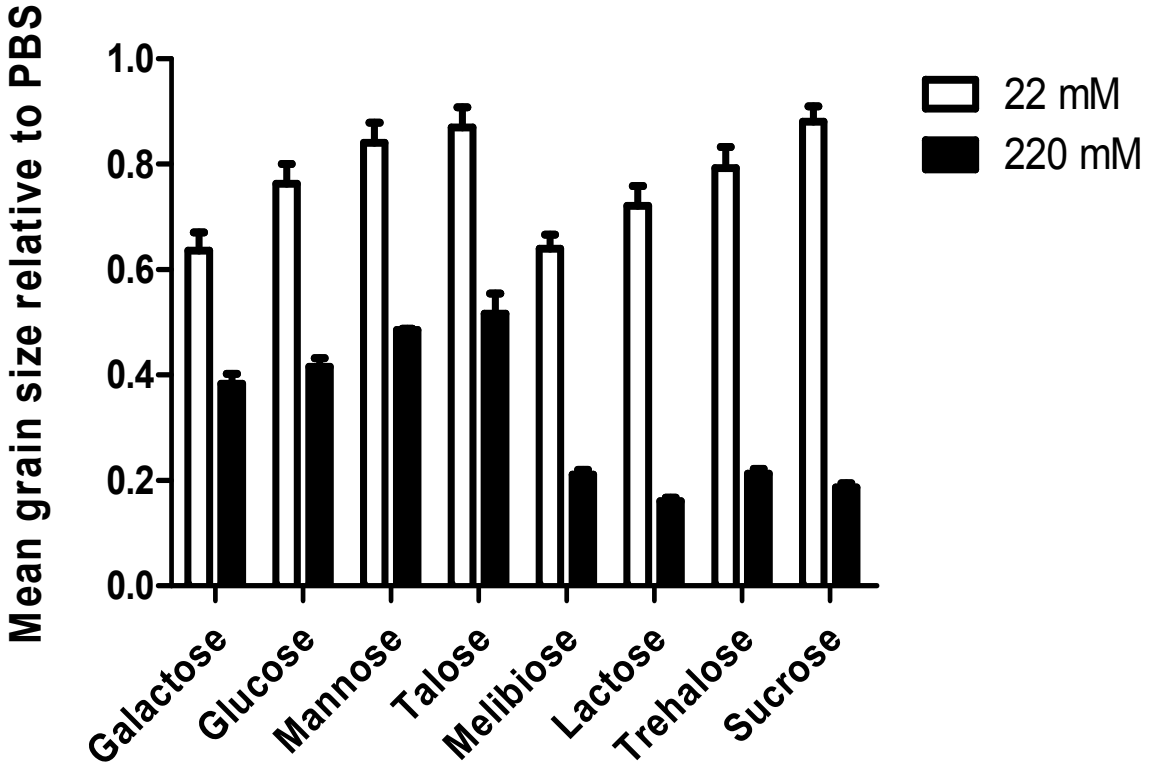
Chapter 3: Results

3.1 Measuring mono- and disaccharides ice recrystallization inhibition activity

Four monosaccharides (D-galactose, D-glucose, D-mannose, D-talose) and four disaccharides (D-melibiose, D-lactose, D-trehalose and D-sucrose) were measured for their IRI activity at two different concentrations, 22 mM and 220 mM concentrations in PBS. A minimum of 3 replicates were performed for each carbohydrate at 22 mM and 220 mM. Carbohydrate concentrations above 220mM were not assessed because the viscosities of those solutions were too high for the splat-cooling assay to accurately measure the IRI activities. The two different concentrations of carbohydrates resulted in notable differences in the mean grain sizes observed, and hence their IRI activity.

First, both mono- and disaccharides resulted in smaller mean grain sizes at 220 mM concentration compared to 22 mM, suggesting that carbohydrate concentration influences IRI activity. The IRI activity of monosaccharides at 220 mM is approximately two times more active than at 22 mM. Secondly, the relative IRI activities of disaccharides compared to monosaccharides are similar at 22mM but different at 220mM. The IRI activities of disaccharides are almost up to three times greater than monosachharides at 220mM, but at 22mM concentrations, these differences were not found. Significant differences between IRI activities of carbohydrates at the same concentration and between the two different concentrations were not calculated due to the low number of replicates performed.

Figure 4. Ice recrystallization inhibition activity of mono- and disaccharides at concentrations of 22 mM (white bars) and 220 mM (black bars). Y-axis represents the % of the mean grain size (MGS) of ice crystals in the solution relative to a containing only phosphate buffered saline (PBS). A minimum of 3 replicates were measured for each carbohydrate at 22 mM and 220 mM. Significant differences in the IRI activity between samples were not calculated due to the low number of replicates.

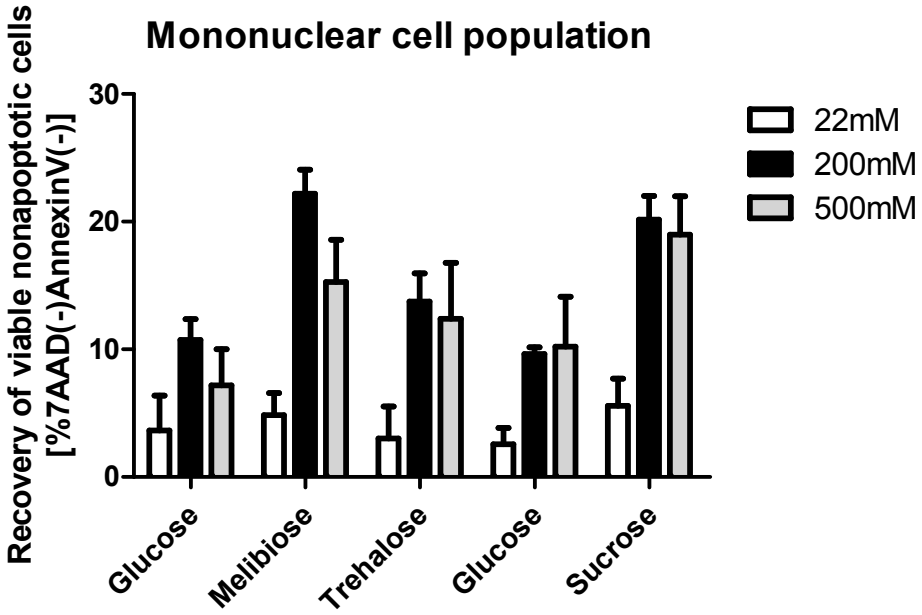

Mean grain size relative to PBS
1.0
0.8
0.6
0.4
0.2
0.0
22 mM
220 mM
Galactose
Glucose
Mannose
Talose
Melibiose
Lactose
Trehalose
Sucrose

3.2 Determining the optimal carbohydrate concentration for sugar cryopreservation of umbilical cord blood

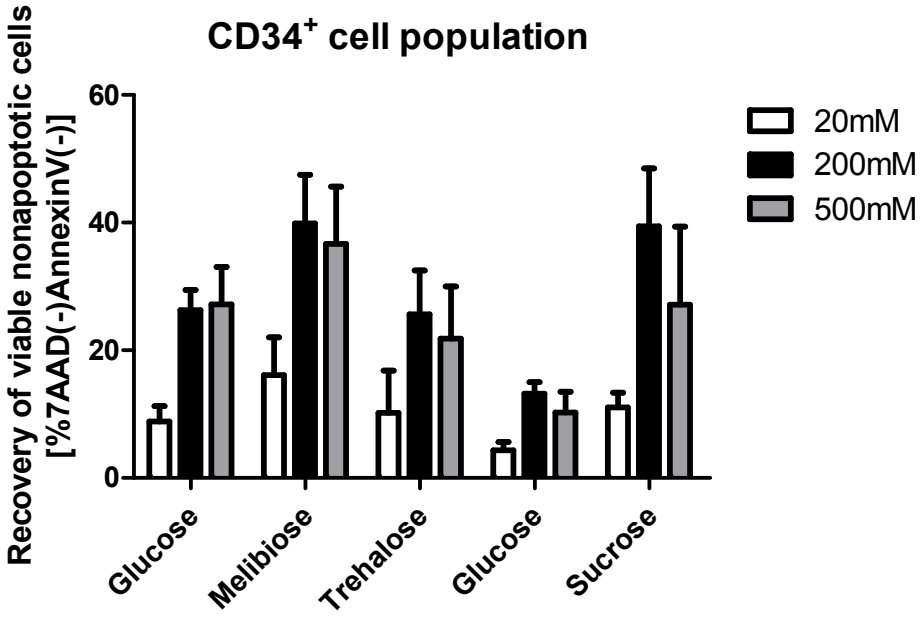
Optimal carbohydrate concentrations for studying the relationship between carbohydrate mediated IRI activity and recovery of viable and nonapoptotic cells were determined by cryopreserving UCB in 3 different concentrations (20mM, 200mM and 500mM) of the 5 sugars used in the study. 2 replicates were measured for each sugar at the 3 different concentrations. In general, cryopreservation of UCB units in 20mM of sugar resulted in low yields of viable nonapoptotic ($7AAD^{-ve}$/ Annexin-V^{-ve}) MNCs (range 2-6%) and CD34+ progenitors (range 4-16%). Cryopreservation of UCB units at higher concentrations of carbohydrates (200mM and 500mM) resulted in improved yields of viable nonapoptotic MNCs (range 10-22%) and $CD34^{+}$ cells (range 10-40%) (See figure 2). Cryopreservation with sugars at 200mM and 500mM generally provided similar yields of viable nonapoptotic MNCs (10-20% for 200mM sugar vs 8-19% for 500mM) and $CD34^{+}$ cells (13-40% for 200mM sugar vs 10-37% for 500mM). Due to the low number of replicates measured for each carbohydrate and concentration, no significant difference in the recovery of nonapoptotic cells between the 200mM and 500mM carbohydrate conditions can be assumed. Because sugar concentrations of 500mM did not provide any increases in recovering viable nonapoptotic MNC or $CD34^{+}$ cells, and was difficult to assess for IRI activity, 200mM was selected as the optimal concentration for cryopreservation to avoid unneeded excess use of sugars.

Figure 5. Yield of viable nonapoptotic MNCs (**A**) and $CD34^+$ cells(**B**) in UCB units following cryopreservation in galactose, melibiose, trehalose, glucose and sucrose at 20mM, 200mM and 500mM. Cryopreservation at 20mM resulted in low yields of nonapoptotic MNCs and $CD34^+$ cells compared to 200mM and 500mM (n=2 for each sugar at 20mM,200mM and 500mM). Use of 200mM sugars appeared to be the optimal concentration since cryopreservation in 500mM generally resulted in similar recoveries of non-apoptotic cells as 200mM, and 500mM IRI activity could not be measured with the splat cooling assay. increase yield of
viable nonapoptotic $CD34^+$ and MNCs.

A


Mononuclear cell population
Recovery of viable nonapoptotic cells
[%7AAD(-)AnnexinV(-)]
30
20
10
0
22mM
200mM
500mM
Glucose
Melibiose
Trehalose
Glucose
Sucrose

B


CD34+ cell population
Recovery of viable nonapoptotic cells
[%7AAD(-)AnnexinV(-)]
60
40
20
0
20mM
200mM
500mM
Glucose
Melibiose
Trehalose
Glucose
Sucrose

3.3 IRI activity of carbohydrates and the yield of viable MNCs and $CD34^+$ cells

UCB units were cryopreserved in 200mM of each carbohydrates and then thawed either in a rapid manner (thawed in a 37 ºC water bath for approximately 1 minute) to minimize ice recrystallization or slow manner (air thawed at room temperature for approximately 6 minutes) to exacerbate the occurrence of ice recrystallization. A concentration of 200mM carbohydrate was chosen because IRI activity of carbohydrate concentrations greater than 220mM could not be measured accurately with the splat cooling assay and 220mM carbohydrates appeared to possess greater IRI activity compared to 22mM.

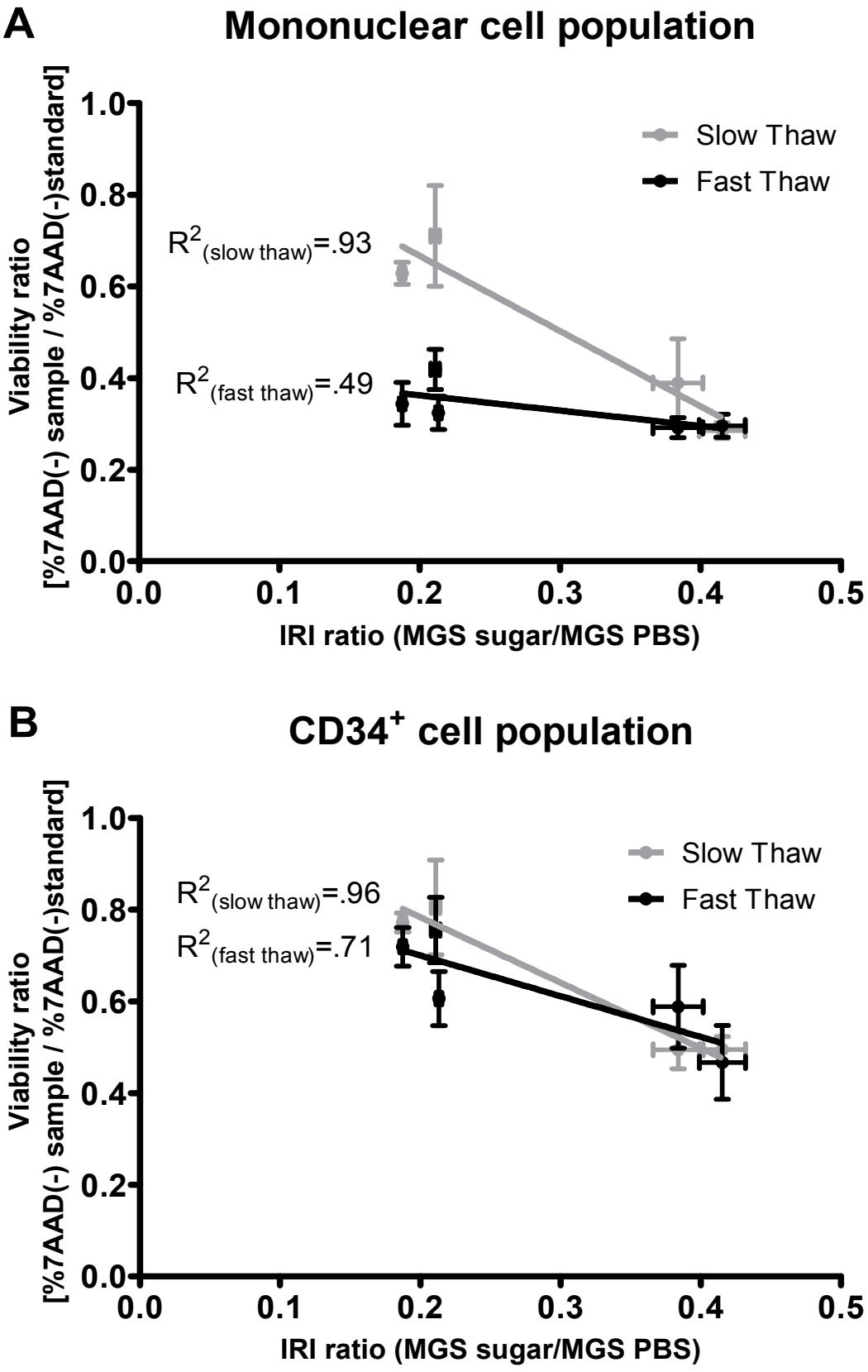
Recovery of viable cells was then assessed by flow cytometry for exclusion of 7AAD. When UCB units were cryopreserved using carbohydrates with potent IRI and thawed under conditions of high recrystallization injury (i.e. thawing slowly at ambient temperature), we observed an increase in the yield of viable MNCs and of viable $CD34^+$ cells (see Figure 3). In particular, IRI activity of carbohydrates correlated significantly with improved yield of viable MNCs (r^2=0.93, p=0.004 for slope different than null hypothesis that slopes are not significantly non zero) and greater yield of viable $CD34^+$ cells (r^2=0.96, p=0.019).

The effect of carbohydrate IRI activity on the yield of total viable MNCs was less apparent under conditions of low recrystallization injury (i.e. thawing rapidly in a 37 ºC

water bath) (r^2=0.49) although a trend toward improved yield of viable $CD34^+$ cells was still observed (r^2=0.705, p=0.07 for the null hypothesis) (see Figure 3).

When UCB units were thawed under more clinically relevant conditions of low recrystallization injury (i.e. thawing rapidly in a 37˚C water bath), both monosaccharides and disaccharides, at 200 mM in the absence of 5% DMSO, produced yields of viable MNCs that were low in comparison to cells cryopreserved under standard conditions containing 5% DMSO only (ranging from 22 ± 4.8% of input cells for glucose to 36 ± 3.4% for melibiose, compared to 79 ± 3.8% for 5% DMSO, p<0.0001). The yield of viable $CD34^+$ cells, however, was greater than the yield of viable total MNCs for all carbohydrate solutions tested (from 40% ± 6.5% for glucose to 61% ± 8.1% for melibiose, p=0.033 when comparing the mean recovery of viable $CD34^+$ cells to mean yield of viable total MNCs for all samples). The yield of viable $CD34^+$ cells was not different from the viability of total MNCs in the control solution containing 5% DMSO alone (88% ± 3.8% vs. 79 ± 3.8%, p=NS).

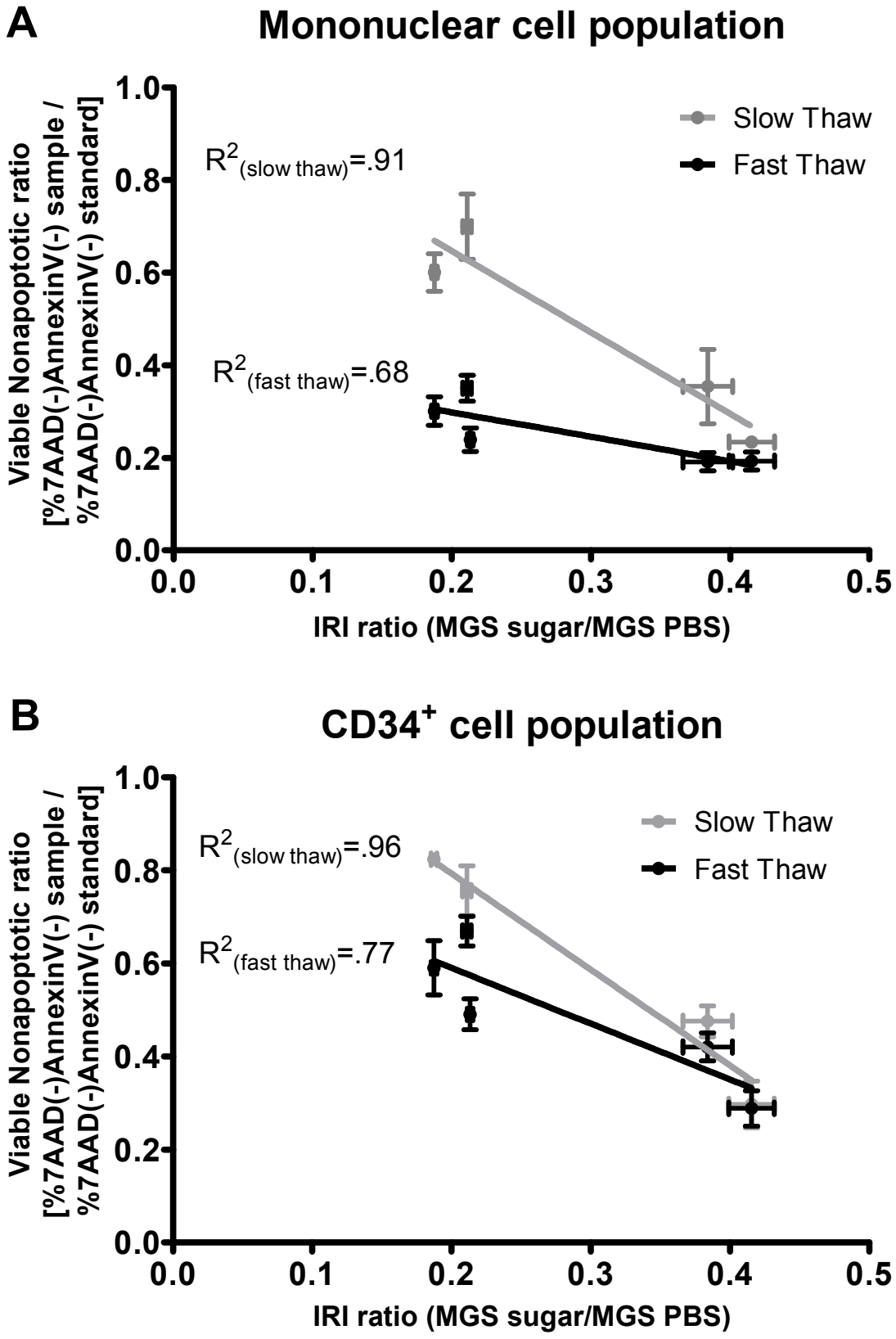
Figure 6. Yield of viable cells vs. IRI activity for MNCs (**A**) and $CD34^+$ cells (**B**) UCB MNCs were cryopreserved with 200 mM (w/v) of the carbohydrate and analyzed post-thaw for their viability by exclusion of 7-AAD. Samples with each carbohydrate were thawed in either a 37°C water bath for the fast thaw (Black lines, n=5) or at room temperature for the slow thaw (Grey lines, n=2). Resulting viability ratios were then plotted against the IRI activity of the corresponding carbohydrate where MGS represents the mean grain size of ice crystals in a solution of carbohydrate or phosphate buffered saline (PBS). A minimum of 3 replicates for each carbodhyrate was measured for the determination of IRI activity.


A
Mononuclear cell population
Viability ratio
[%7AAD(-) sample / %7AAD(-)standard]
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)
Slow Thaw
Fast Thaw
R2(slow thaw)=.93
R2(fast thaw)=.49
B
CD34+ cell population
Viability ratio
[%7AAD(-) sample / %7AAD(-)standard]
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)
Slow Thaw
Fast Thaw
R2(slow thaw)=.96
R2(fast thaw)=.71

3.4 IRI activity of carbohydrates and the yield of nonapoptotic MNCs and $CD34^+$ cells

Apoptosis, as measured by annexin-V, was measured in combination with viability in the cryopreserved UCB samples. Overall, apoptosis was found to be affected by thawing rates and appears to be modulated by the presence of carbohydrates with greater IRI activity (Figure 4). In general, under slow thawing which produces a larger amount of ice recrystallization, carbohydrates with greater IRI activity produced higher yields of viable nonapoptotic MNCs (r^2=0.91, p=0.002) and viable nonapoptotic $CD34^+$ cells (r^2=0.96, p=0.0001), expressed as a ratio in comparison to standard 5% DMSO conditions. The percentage of apoptotic cells recovered was significantly more reduced within the $CD34^+$ population in comparison to MNCs (p=0.006). Similar to measurements of viability, the effect of carbohydrate IRI activity on the yield of viable nonapoptotic MNCs and $CD34^+$ cells were less apparent under conditions of low recrystallization injury (i.e. thawing rapidly in a 37 ºC water bath). However, there was still a trend towards improved yield of viable nonapoptotic MNCs (r^2=0.68, p= 0.088 for null hypothesis) and $CD34^+$ cells (r^2=0.77, p=0.05 for the null hypothesis) (see Figure 4). The percentage of viable nonapoptotic total MNCs cryopreserved with IRI potent carbohydrates, but without 5% DMSO, and thawed at 37ºC is low (8.2 ± 2.2% for glucose to 22% ± 2.7% for melibiose) in comparison to samples cryopreserved in standard 5% DMSO only (60 ± 9.1%, p=0.0005 in comparison with carbohydrates).

Figure 7. **A**. Yield of viable non-apoptotic (Annexin V^{-ve} / $7AAD^{-ve}$) cells vs. IRI activity for MNCs (**A**) and $CD34^{+}$ cells(**B**). MNCs from UCB samples were cryopreserved with 200 mM (w/v) of the carbohydrate and were analyzed (**A**) along with the $CD34^{+}$ population (**B**) post-thaw for apoptosis using annexin-V. Samples with each carbohydrate were thawed in either a 37°C water bath for the fast thaw (Black lines, n=5) or at room temperature for the slow thaw (Grey lines, n=2). Resulting viable non-apoptotic ratios were then plotted against the IRI activity of the corresponding carbohydrate where MGS represents the mean grain size of ice crystals in a solution of carbohydrate or phosphate buffered saline (PBS).


A
Mononuclear cell population
Viable Nonapoptotic ratio
[%7AAD(-)AnnexinV(-) sample /
%7AAD(-)AnnexinV(-) standard]
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)
Slow Thaw
Fast Thaw
R2(slow thaw)=.91
R2(fast thaw)=.68
B
CD34+ cell population
Viable Nonapoptotic ratio
[%7AAD(-)AnnexinV(-) sample /
%7AAD(-)AnnexinV(-) standard]
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)
Slow Thaw
Fast Thaw
R2(slow thaw)=.96
R2(fast thaw)=.77

3.5 *Carbohydrates with greater IRI activity preserve colony forming unit capacity of UCB samples.*

Colony forming unit (CFU) assays were also performed to provide relevant insight into the relationship between carbohydrate mediated ice recrystallization inhibition and preservation of functional capacity. Example images of the colonies enumerated and their plate densities following cryopreservation in 5% DMSO or 200mM carbohydrates are illustrated in Figure 7.

We observed a strong correlation between greater preservation of CFU activity and IRI activity of the carbohydrates used for cryopreservation. The most active ice recrystallization-inhibitors preserved the CFU-forming activity to a greater extent (r^2=0.92, p=<0.0001) (Figure 5A). Following cryopreservation and fast thawing, the range of total CFUs cryopreserved in carbohydrates was lower (5.7 ± 2.2 per 10^4 cells for glucose to 30 ± 3.5 for melibiose) than the average number of total colonies obtained from samples cryopreserved in 5% DMSO only (76 ± 10 per 10^4 cells (p=0.001)). We observed similar results for the most dominant colony forming unit CFU-GM (Figure 5B).

Figure 8. Typical colony-forming unit (CFU) assays (40x magnification) revealing colony densities from CB units cryopreserved in (A) 5% DMSO, (B) 200 mM melibiose and (C) 200 mM galactose. (D) Mean number (±SEM) total colonies formed following cryopreservation in 200 mM carbohydrate solution or 5% DMSO. (E–H) Typical CFU colonies observed in methylcellulose assays after cryopreservation and thawing of cord blood. CFUs were identified by morphology and enumerated using an inverted microscope to discriminate erythroblast-forming units (BFU-E) (E), granulocyte/macrophage (CFU-GM) (F), granulocyte/erythroid/macrophage/monocyte (CFU-GEMM) (G) and erythroid (CFU-E) (H) colonies.

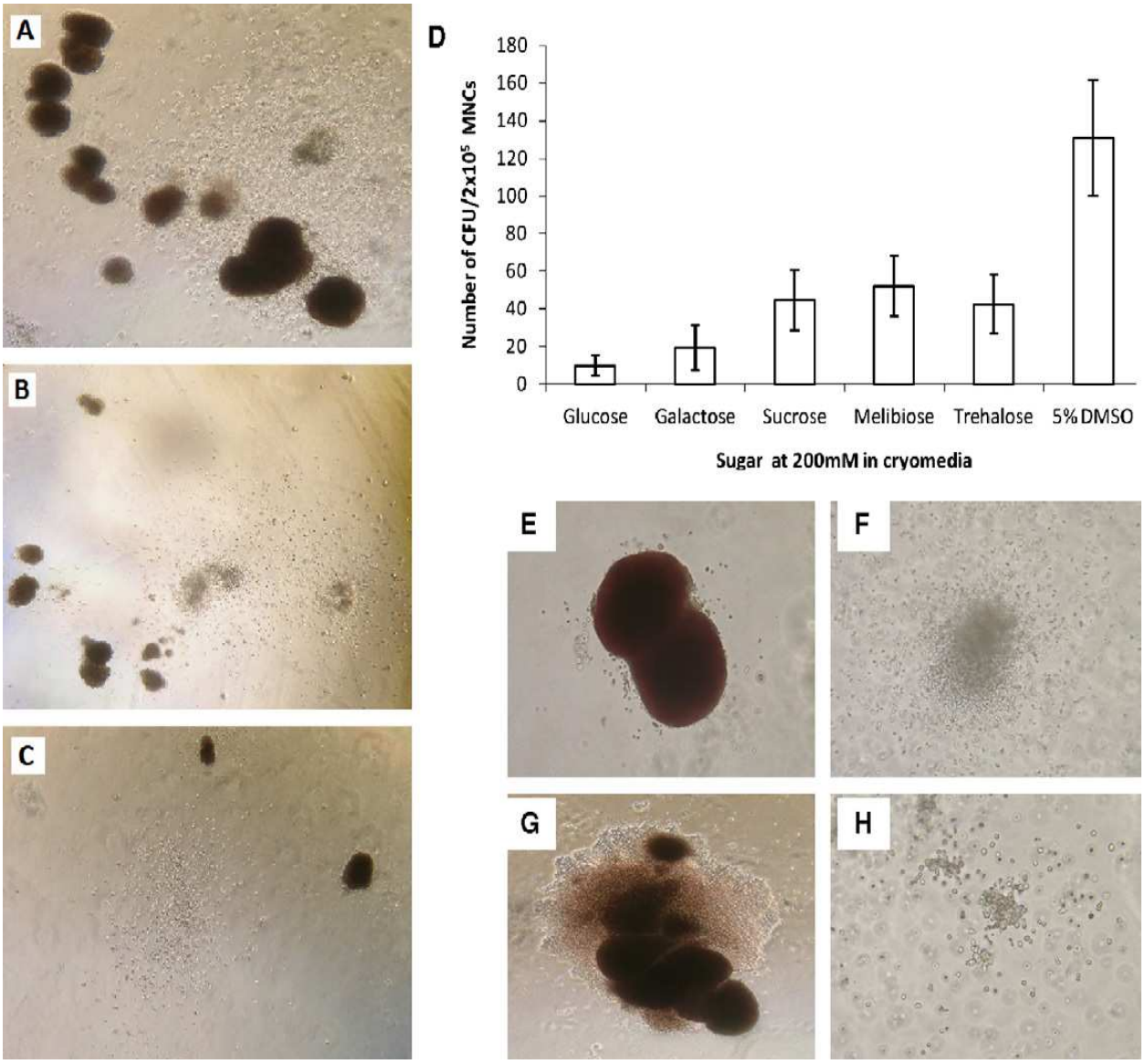
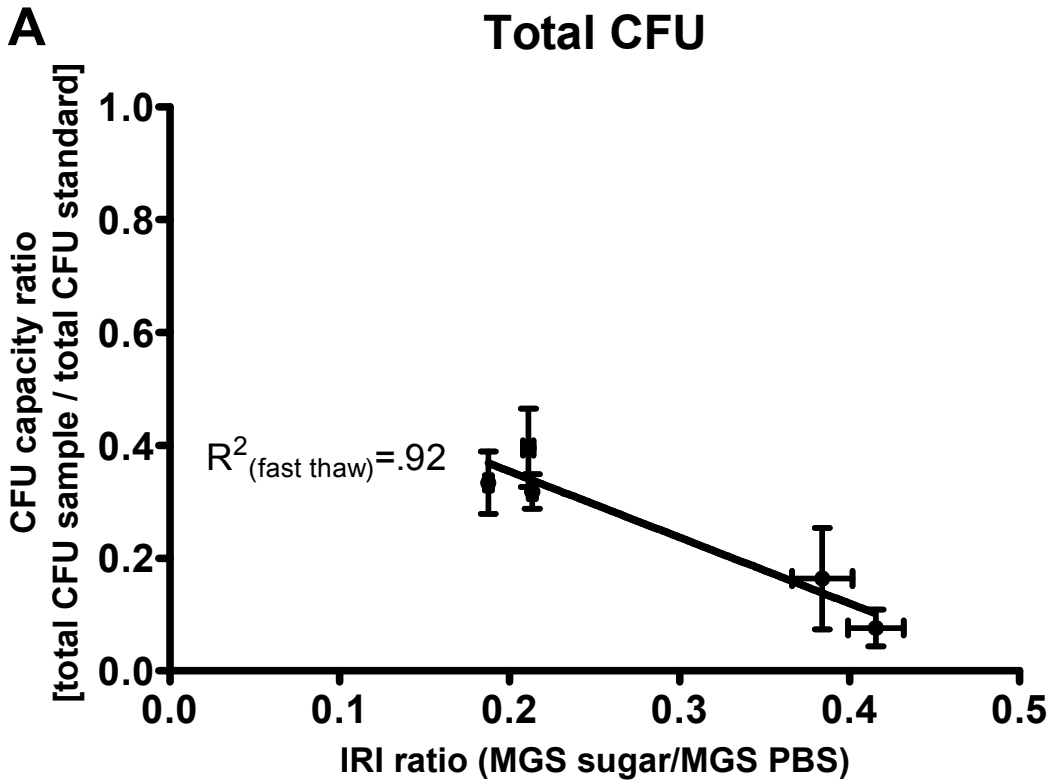
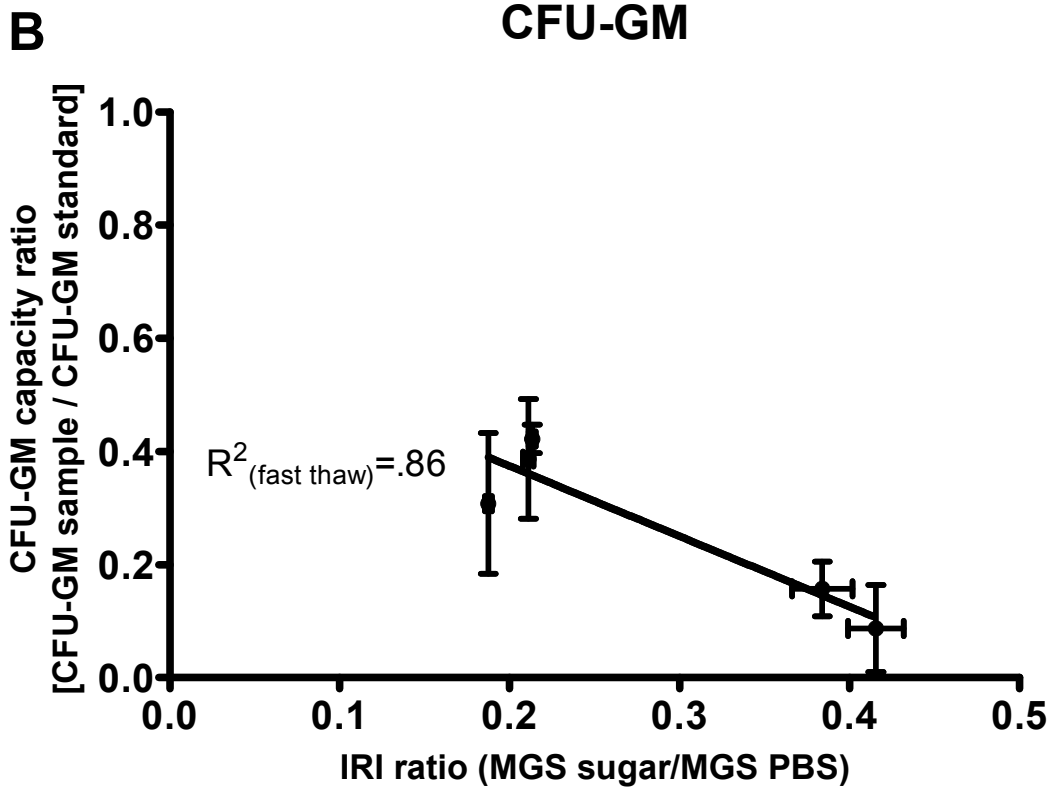

A
B
C
D
Number of CFU/2x10^5 MNCs
180
160
140
120
100
80
60
40
20
0
Glucose
Galactose
Sucrose
Melibiose
Trehalose
5% DMSO
Sugar at 200mM in cryomedia
E
F
G
H

Figure 9. Colony Forming Unit capacity vs. IRI activity. UCB MNCs were cryopreserved with 200 mM (w/v) of the carbohydrate, fast-thawed and cultured for 2 weeks in semi-solid methylcellulose media and total colonies (n=3 for each sugar) (**A**) and CFU-GM (n=3 for each sugar) (**B**) were enumerated. Samples with each carbohydrate were thawed in a 37°C water bath for the fast thaw (Black lines, n=5) The total colonies obtained from each sample are expressed relative to CFU number from a control sample from the same UCB unit which was cryopreserved with 5% DMSO (v/v). Resulting CFU capacity ratios were then plotted against the IRI activity of the corresponding carbohydrate where MGS represents the mean grain size of ice crystals in a solution of carbohydrate or phosphate buffered saline (PBS).


A
Total CFU
CFU capacity ratio
[total CFU sample / total CFU standard]
1.0
0.8
0.6
0.4
0.2
0.0
R2(fast thaw)=.92
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)


B
CFU-GM
CFU-GM capacity ratio
[CFU-GM sample / CFU-GM standard]
1.0
0.8
0.6
0.4
0.2
0.0
R2(fast thaw)=.86
0.0
0.1
0.2
0.3
0.4
0.5
IRI ratio (MGS sugar/MGS PBS)

3.6 Delayed engraftment and case control characteristics

A nested case control study was designed to assess if cold induced apoptosis was was associated with delayed neutrophil engraftment after autologous HSCT. This type of study design was selected over a retrospective cohort study because retrospective cohort studies require analyzing all patients in a cohort, whereas nested case control studies require analyzing only the patients that developed the disease of interest and a specified number of matched controls within the same cohort that did not develop the disease of interest. Overall, the nested case control study design was utilized to minimize cost and time associated with a full retrospective cohort study.

326 lymphoma patients were identified who received autologous peripheral blood stem cell grafts with a target $CD34^+$ progenitor dose $\geq 2.0 \times 10^6$ cells/kg, with 14 patients out of the 326 experiencing delayed neutrophil engraftment. PBSC graft aliquots from 13 out of the 14 delayed engraftment cases were available for analysis. PBSC graft aliquots from 28 patients in the original cohort that did not experience delayed neutrophil engraftment were also selected for analysis as matched controls. PBSC graft aliquots from 22 out of the 28 matched controls were available for analysis. These 22 matched controls were matched for age, male/female ratio, percentage of hodgkins lymphoma cases, and mean quantity of $CD34^+$ progenitors and MNC harvested/kg. The mean time to initial neutrophil engraftment (1^{st} of 3 consecutive days with greater than 0.5×10^9 neutrophil/L) and the mean length of stay in hospital (LOS), however, was greater in the delayed engraftment case group compared to the matched control group *(table 1)*. Additionally, a small number of patients had $< 2.0 \times 10^6$ $CD34^+$

progenitors/kg at the time of collection. The number was similar between the delayed engraftment cases (2/13, 15%) and control group (2/22, 9%, p=NS), and no significant difference was observed between cases and controls with regard to the number of patients exceeding the optimal threshold of 5.0×10^6 $CD34^+$ progenitors/kg at the time of collection (p=NS) *(table 2)*.

Table 1. Characteristics of study cases with delayed neutrophil engraftment and matched controls. Controls were matched with delayed engraftment cases for gender ratio, age, $CD34^+$ progenitors and MNCs collected / kg patient weight, type of lymphoma (hodgkins or nonhodgkins). Delayed engraftment cases required a significantly longer time to reach an average neutrophil count of > 0.5×10^9 cells / L blood and required a length of stay (LOS) in hospital compared to matched controls.

Delayed engraftment vs control characteristics			
	Delayed engraftment cases	Matched controls	p
Number Patients (N)	13	22	
Age (mean±SD)	48±14	47±15	0.8
gender (M/F)	7/6	12/10	N/A
Hodgkins Lymphoma / NHL	2/11	3/19	N/A
$CD34^+$ progenitors / kg harvested	$(3.7\pm2.0) \times 10^6$	$(4.4\pm1.6) \times 10^6$	0.26
MNCs/kg harvested	$(7.9\pm3.5) \times 10^8$	$(8.1\pm4.4) \times 10^8$	0.87
days to ANC > 0.5 (mean SD)	17.6±6.6	11.9±3.2	0.002
LOS (d, mean +/- SD)	31.2±11.4	22.6±5.9	0.006

Table 1. Proportion of study cases and controls reaching the minimum $CD34^+$ progenitor dose threshold ($2.0x10^6$ cells/kg) and the optimal $CD34^+$ progenitor dose threshold ($5.0x10^6$ cells/kg) at collection, after thawing and considering only viable $CD34^+$ cells, and after thawing and considering only viable nonapoptotic $CD34^+$ cells.

Proportion of patients meeting CD34 threshold dose			
	Delayed engraftment cases	Matched Controls	P
Patients receiving > minimum CD34 dose			
At collection	11 (85%)	20 (91%)	0.6
Post thaw, viable	10 (77%)	17 (77%)	1.0
Post thaw, viable nonapoptotic	6 (46%)	17 (77%)	0.08
Patients receiving > optimal CD34 dose			
At collection	2(17%)	8 (36%)	0.3
Post thaw, viable	2 (17%)	6 (27%)	0.7
Post thaw, viable nonapoptotic	2 (17%)	3 (14%)	1.0

3.7 The association between viability and delayed neutrophil engraftment in PBSC grafts

The percentage and absolute number of cells excluding 7AAD transplanted into the delayed engraftment and control patients were assessed to determine if loss of viability was associated with reduced functionality in a transplant setting. Patients with delayed neutrophil recovery and controls had similar percentages of viable MNCs and viable $CD34^+$ progenitors following processing and thawing (see Figure 7). In general, PBSC grafts maintained high levels of cell viability in both the MNC population (91.0 ± 1.3% for cases vs 91.6 ± 0.7 % for controls, p=0.7) and the $CD34^+$ progenitor population (84.6 ± 2.7% for cases vs. 85.5 ± 1.4% for controls, p=0.8) after thawing (see Figure 6). In addition, the absolute number of viable MNCs and $CD34^+$ progenitors available for reinfusion and calculated based on patients' weight was calculated to determine the actual dose of cells for each patient and were similar for cases and controls (see Figure 7). Further, the proportion of patients with delayed neutrophil recovery who received more than 2.0 x 10^6 $CD34^+$ progenitors/kg was similar to the controls (10/13, 77% vs 17/22, 77%, p=NS) and likewise, the proportion of patients receiving more than 5.0 x 10^6 $CD34^+$ progenitors/kg was the same in cases and controls (2 of 13 cases, 17% vs. 6 of 22 controls, 27%, p=NS) (see table 2).

Figure 10. Changes in cell viability and apoptosis after thawing cryopreserved PBSCs. Typical flow cytometry analysis of mononuclear cells (gated from R1 in figure 1) (**A**), $CD34^{+}$ progenitors (gated from R3 in figure 1) (**B**) and $CD34^{+}CD45^{low}ALDH^{high}$ progenitors (gated from R4 in figure 1) (**C**) in freshly collected PBSCs and of mononuclear cells (**D**), $CD34^{+}$ progenitors (**E**) and $CD34^{+}CD45^{low}ALDH^{high}$ progenitors (**F**) after thawing cryopreserved PBSCs. The bottom left and bottom right quadrants of all dot plots represent the viable cells that exclude 7AAD while the bottom right quadrants specifically represents viable apoptotic cells that bind AnnexinV-PE but exclude 7AAD, while the bottom left quadrants specifically represent viable nonapoptotic cells that exclude 7AAD and didn't bind Annexin-V.

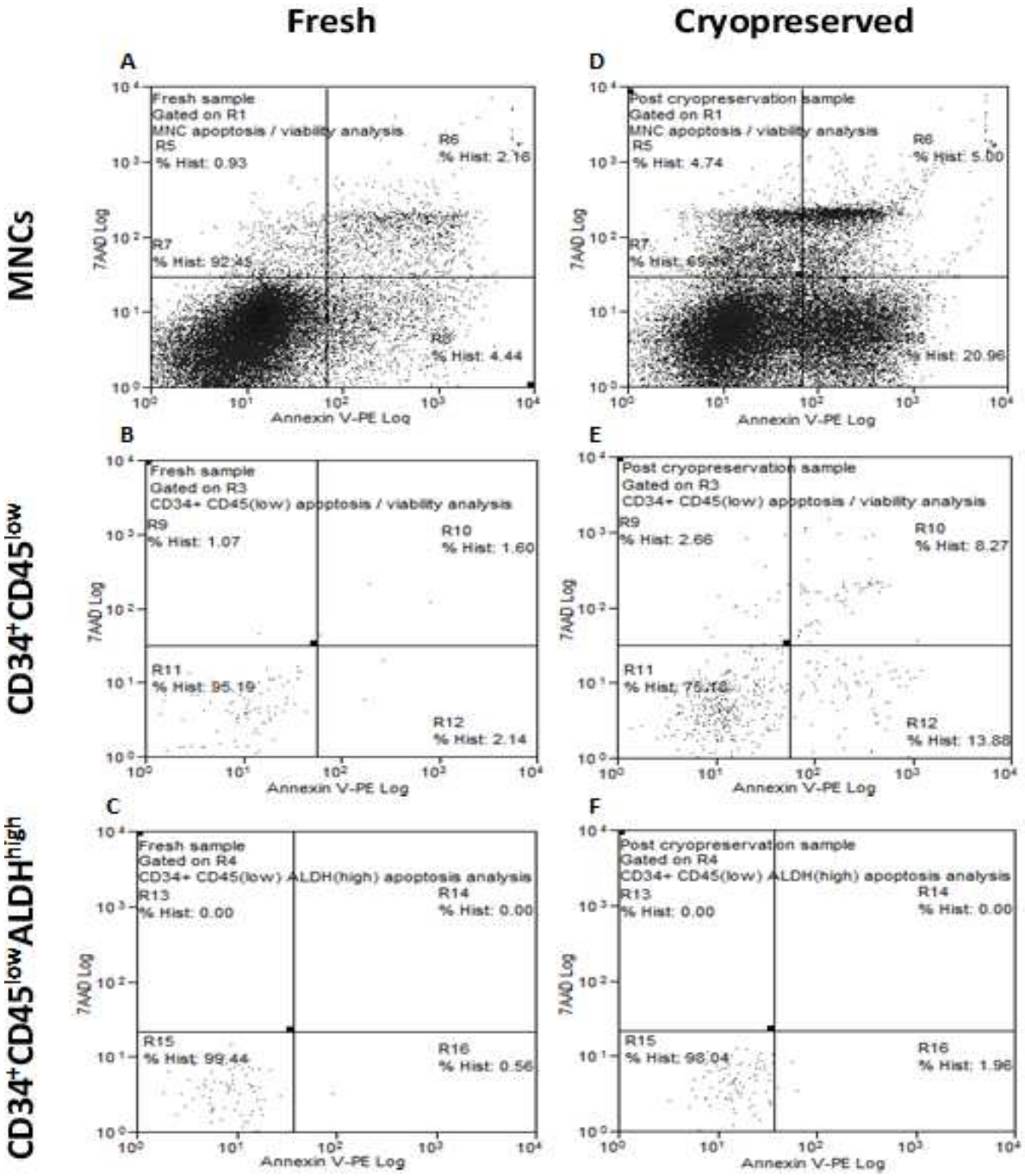
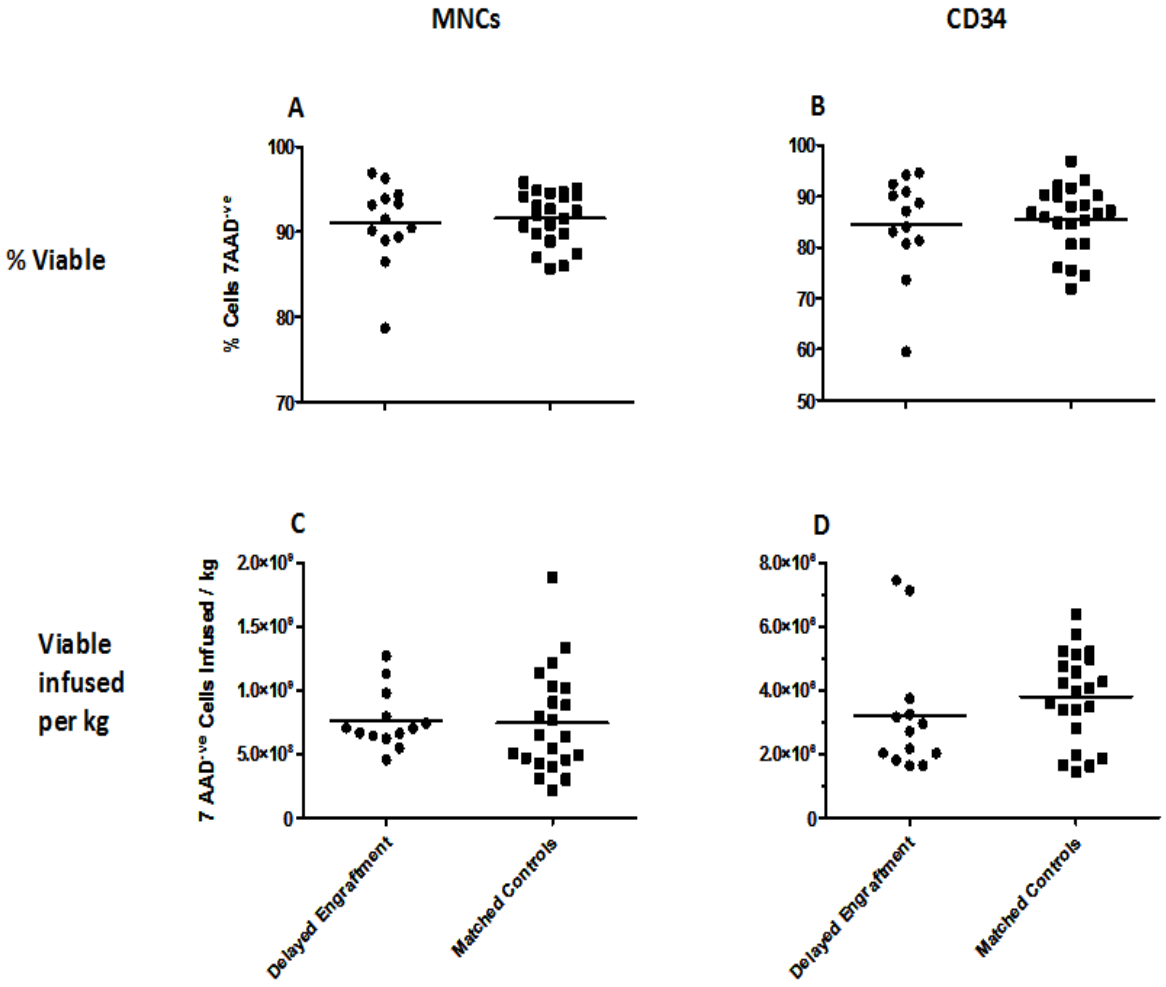

Fresh
Cryopreserved
MNCs
CD34+CD45low
CD34+CD45lowALDHhigh
A
Fresh sample
Gated on R1
MNC apoptosis / viability analysis
R5
% Hist: 0.93
R6
% Hist: 2.16
R7
% Hist: 92.45
R8
% Hist: 4.44
7AAD Log
Annexin V-PE Log
D
Post cryopreservation sample
Gated on R1
MNC apoptosis / viability analysis
R5
% Hist: 4.74
R6
% Hist: 5.00
R7
% Hist: 69.30
R8
% Hist: 20.96
7AAD Log
Annexin V-PE Log
B
Fresh sample
Gated on R3
CD34+ CD45(low) apoptosis / viability analysis
R9
% Hist: 1.07
R10
% Hist: 1.60
R11
% Hist: 95.19
R12
% Hist: 2.14
7AAD Log
Annexin V-PE Log
E
Post cryopreservation sample
Gated on R3
CD34+ CD45(low) apoptosis / viability analysis
R9
% Hist: 2.66
R10
% Hist: 8.27
R11
% Hist: 75.13
R12
% Hist: 13.88
7AAD Log
Annexin V-PE Log
C
Fresh sample
Gated on R4
CD34+ CD45(low) ALDH(high) apoptosis analysis
R13
% Hist: 0.00
R14
% Hist: 0.00
R15
% Hist: 99.44
R16
% Hist: 0.56
7AAD Log
Annexin V-PE Log
F
Post cryopreservation sample
Gated on R4
CD34+ CD45(low) ALDH(high) apoptosis analysis
R13
% Hist: 0.00
R14
% Hist: 0.00
R15
% Hist: 98.04
R16
% Hist: 1.96
7AAD Log
Annexin V-PE Log

Figure 11. Comparison between study cases with delayed neutrophil engraftment and matched controls regarding the percentage of infused MNC (**A**) and $CD34^+$ progenitors (**B**) that were viable, defined as 7-AAD-negative by flow cytometry, and regarding the absolute number of viable MNC (**C**) and $CD34^+$ progenitors (**D**) infused per kilogram recipient weight. Differences in mean values between cases and controls were not significantly different ($p>0.05$).


MNCs
CD34
A
B
% Viable
% Cells 7AAD-ve
100
90
80
70
100
90
80
70
60
50
C
D
Viable infused per kg
7 AAD-ve Cells Infused / kg
2.0×10⁹
1.5×10⁹
1.0×10⁹
5.0×10⁸
0
8.0×10⁸
6.0×10⁸
4.0×10⁸
2.0×10⁸
0
Delayed Engraftment
Matched Controls
Delayed Engraftment
Matched Controls

3.8 Apoptosis in PBSC grafts as predictor of delayed neutrophil engraftment

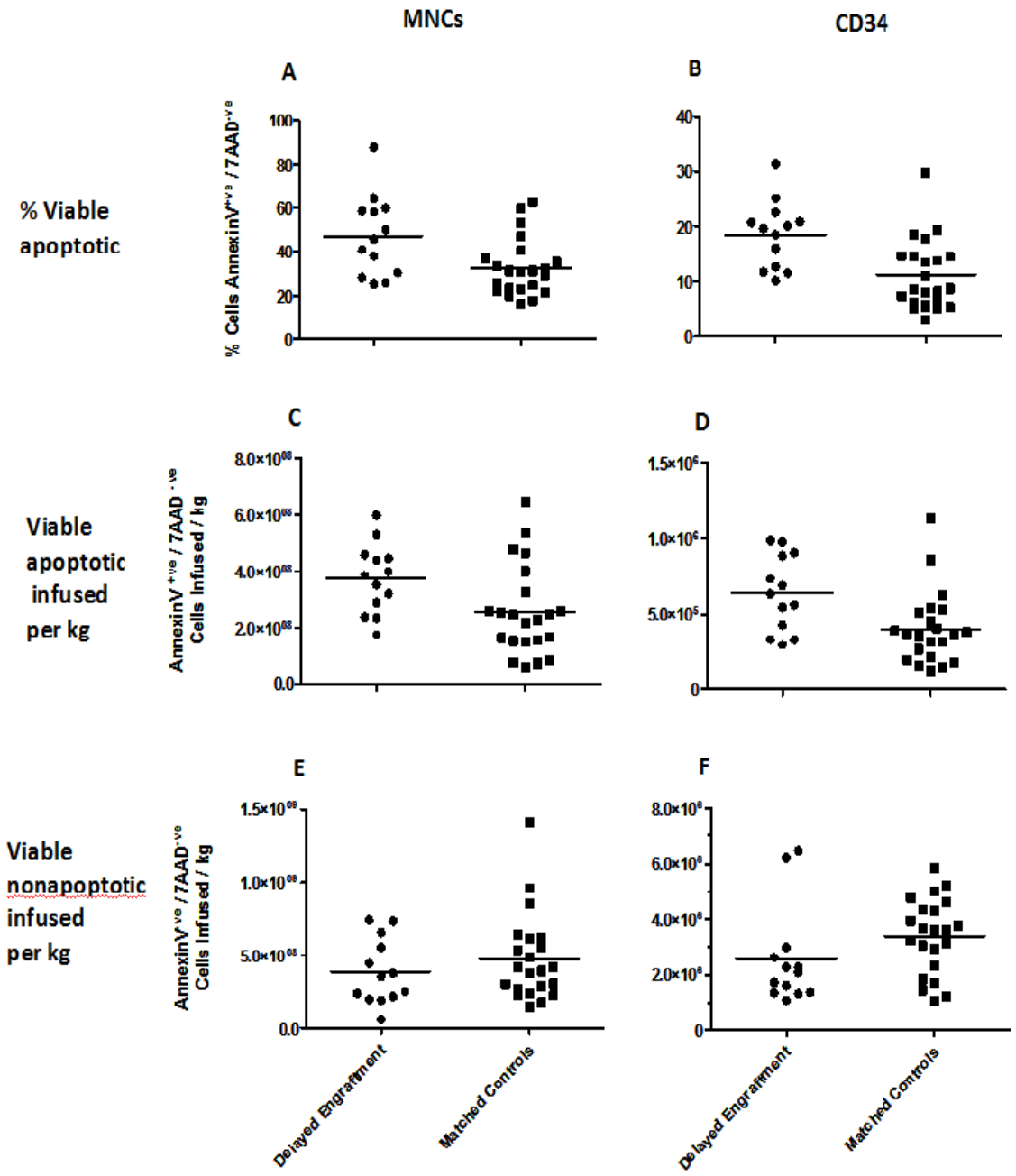
The percentage and absolute number of cells binding Annexin that were transplanted into the delayed engraftment and control patients were also assessed to determine if cold-induced apoptosis was associated with reduced functionality in a transplant setting. Although similar high levels of viable MNCs and $CD34^+$ progenitors measured with 7AAD exclusion alone were maintained during cryopreservation and thawing in both cases and controls, we observed significant levels of apoptosis measured using multicolour flow cytometry ranging from 16 – 88% of MNCs and 3 – 30% of the $CD34^+$ progenitor fraction in the entire cohort of study cases and controls. In contrast, fresh PBSC grafts sampled immediately following collection and processing but prior to freezing had low levels of apoptotic MNCs (6.6 ± 0.8%, n=3) and CD34 progenitors (1.0 ± 0.2%, n=3). In both fresh and cryopreserved samples, the $CD34^+ALDH^{Hi}$ population identified using flow cytometry was found to be entirely viable and nonapoptotic (>99.5% in all samples, see Figure 6).

In contrast to our findings with regard to overall cell viability using the exclusion dye 7AAD, study cases with delayed neutrophil engraftment received PBSC grafts with significantly greater mean percentage of viable apoptotic MNCs compared with controls (47.2 ± 5.1% vs 32.6 ± 2.7% p=0.01) and CD34+ progenitors (18.5 ± 1.7 vs 11.2 ± 1.4 p=.002) (see Figure 8). In addition to receving greater mean percentages of viable apoptotic cells, delayed neutrophil engraftment cases received significantly greater mean absolute numbers of viable apoptotic MNCs ($3.8 \pm 0.3x10^8$ vs $2.6 \pm 0.3x10^8$

MNCs per kg, p=0.03) and $CD34^+$ cells (6.4 ± 0.7x 10^5 vs 4.0 ± 0.5x10^5 $CD34^+$ progenitors/kg, p=0.009) as well. Surprisingly, however, delayed engraftment cases did not receive a significantly greater mean absolute number of viable non-apoptotic MNCs (p=0.4) and CD34+ progenitors (p=0.12) (see Figures 8). Interestingly, there was a trend towards a lower dose of $CD34^+ALDH^{Hi}$ cells/kg in study cases compared with controls (1.1 ± 0.2x10^6 vs 1.7 ± 0.2x10^6 cells/kg, p=0.06).

We observed a trend towards a reduced proportion of study cases with delayed neutrophil engraftment as compared to matched controls that received more than 2.0x10^6 viable and non-apoptotic $CD34^+$ progenitors per kg (6 of 13 cases, 46% vs. 17/22 controls, 77%, p=0.07) (see Table 2) but no significant difference between cases and controls with regards to the proportion of patients receiving more that 5.0 x 10^6 viable non-apoptotic $CD34^+$ progenitors per kilogram.

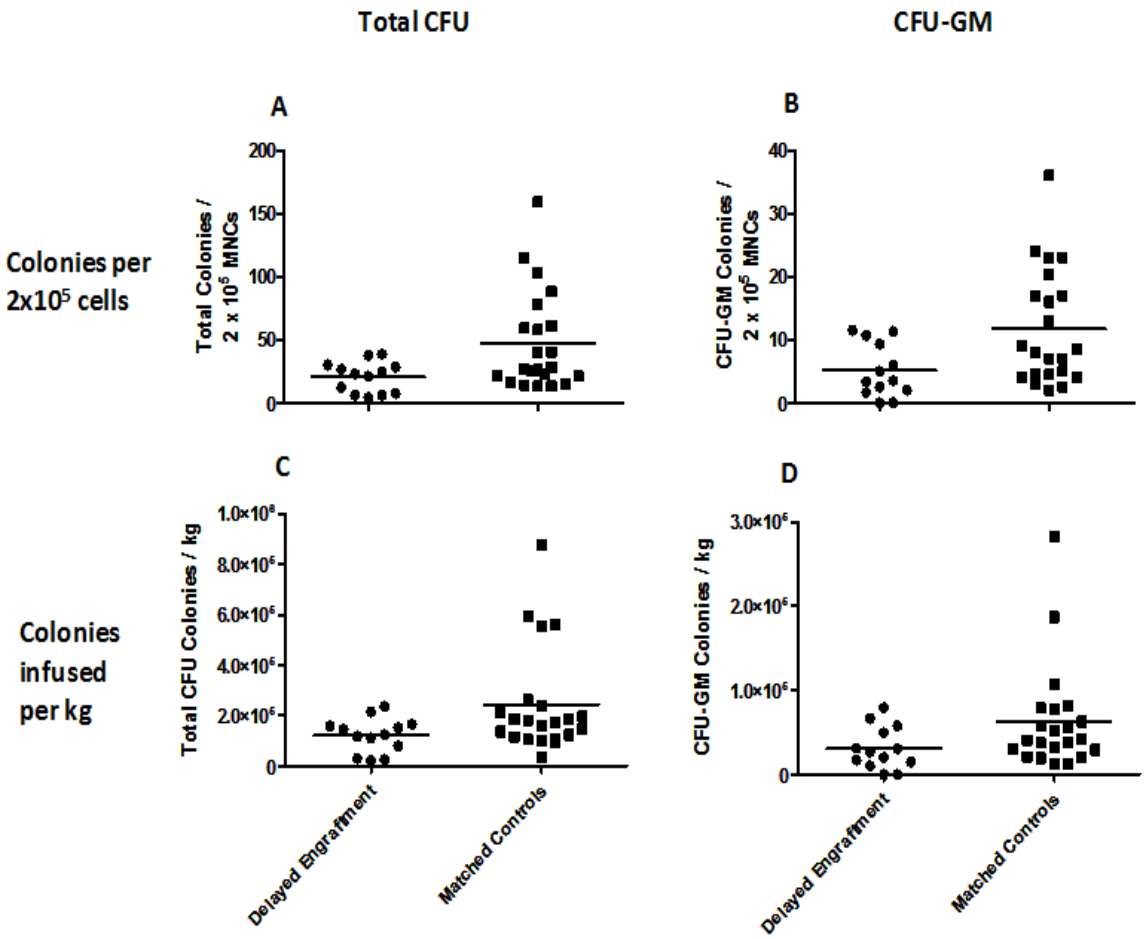
Figure 12. Study cases with delayed neutrophil engraftment had greater percentage of viable apoptotic MNC (**A**) and $CD34^+$ progenitors (**B**) in PBSCs compared with matched controls ($p<0.05$), defined as 7-AAD-negative but Annexin-V-positive events, and greater absolute number of viable apoptotic MNC (**C**) and $CD34^+$ progenitors (**D**) infused per kilogram recipient weight ($p<0.05$). However, no significant difference ($p>0.05$) was observed regarding the absolute number of viable nonapoptotic MNC (**E**) or $CD34^+$ progenitors (**F**) per kilogram recipient weight, defined as 7-AAD-negative and Annexin-V-negative events between cases and controls.


MNCs
CD34
A
B
C
D
E
F
% Viable apoptotic
Viable apoptotic infused per kg
Viable nonapoptotic infused per kg
% Cells AnnexinV+ve / 7AAD-ve
AnnexinV+ve / 7AAD -ve Cells Infused / kg
AnnexinV+ve / 7AAD-ve Cells Infused / kg
Delayed Engraftment
Matched Controls

3.9 CFU levels in PBSC grafts of patients with delayed neutrophil recovery.

Colony-forming units were enumerated after plating thawed mononuclear cells in methylcellulose assays to identify how cryopreservation-associated cell death may be associated with reduced functionality in a transplant setting. PBSC grafts from study cases with delayed neutrophil recovery had reduced total CFU plating efficiencies compared with controls (20.4 ± 3.4 colonies per $2x10^5$ cells vs 47.4 ± 8.4 per $2x10^5$ cells, p=0.022) and reduced dose of total CFUs per kg ($1.2 \pm 0.2x10^5$ colonies / kg vs $2.4 \pm 0.5x10^5$ colonies / kg, p=0.05 see Figure 9). CFU-GM plating efficiencies were also reduced in cases of delayed engraftment (p=0.02, see Figure 9) and there was a trend towards a lower dose of CFU-GMs per kg in study cases compared with controls ($3.1 \pm 0.7x10^4$ CFU-GM / kg vs $6.3 \pm 1.3x10^4$ CFU-GM / kg, p=0.06). The plating efficiency of BFU-E and the quantity of BFU-E / kg was also significantly reduced in cases of delayed engraftment. (See Appendix B)

Figure 13. The plating efficiency of total CFUs (**A**) and CFU-GMs (**B**) are decreased in PBSCs of study cases with delayed neutrophil engraftment in comparison with controls ($p<0.05$). The absolute number of total CFUs (**C**) per kilogram recipient weight is decreased in study cases compared with controls ($p=0.05$) and the absolute number of CFU-GM (**D**) per kilogram recipient weight shows a decreasing trend in study cases compared with controls ($p=0.09$) and


Total CFU
CFU-GM
A
B
C
D
Colonies per 2x10^5 cells
Colonies infused per kg
Total Colonies / 2 x 10^5 MNCs
CFU-GM Colonies / 2 x 10^5 MNCs
Total CFU Colonies / kg
CFU-GM Colonies / kg
Delayed Engraftment
Matched Controls

Chapter 4: Discussion

With respect to the first aspect of the experimental work described in this thesis, we focused on addressing the role of carbohydrate mediated IRI as a mechanism of reducing cryopreservation-associated injury and cell death in UCB. Overall, results from this thesis demonstrate that carbohydrates with greater ice recrystallization-inhibition activity reduce cryopreservation-associated cell death in UCB. Specifically, we observed significant correlations between the IRI activity of various carbohydrates and the yield of viable non-apoptotic cells, including hematopoietic stem cell populations and progenitor CFU function. The effect of carbohydrate IRI was most apparent under conditions that exacerbate ice recrystallization, which supports the notion that carbohydrates mediate cryoprotection through inhibition of ice recrystallization.

Carbohydrate IRI activity also appears specifically relevant for the stem and progenitor cells, as suggested by our results regarding greater yield of viable and non-apoptotic $CD34^+$ cells in comparison with total mononuclear cells. Interestingly, others have reported that some tissue-specific stem and progenitor cells may be very sensitive to cold-induced apoptosis (114, 115). For instance it has been demonstrated that specific hematopoietic stem and progenitor populations such as megakaryocytes may be particularly sensitive to cryopreservation-associated damage in comparison to other blood progenitor populations, according to one previous report (116). Sparrow *et al.*(117) documented the lymphocyte population was even more sensitive to cryopreservation-associated damage compared to the $CD34^+$ population in UCB. In our

studies, we found that mononuclear cells from both UCB and PBSCs were more sensitive to cold-induced apoptosis compared to the $CD34^+$ population.

Models to explain why some cell types may be more susceptible to cold-induced apoptosis are lacking, but influencing factors may include cell membrane characteristics and susceptibility of the membrane to ice recrystallization injury, differences in the capacity for cells to reverse apoptosis pathways and differences in susceptibility to intracellular ice formation. The formation of extracellular ice has been reported to enhance the formation of intracellular ice, either through disruption of the plasma membrane, allowing ice to propagate through the plasma membrane into the cell, or by causing conformational changes in the membrane(9). In either case, ice recrystallization in the extracellular compartment may exacerbate the formation of intracellular ice in cells with membranes more susceptible to damage or conformational changes for the brief period during thawing. This brief intracellular ice may damage mitochondrial membranes, inducing changes in the mitochondrial membrane potential, a mechanism thought to be responsible for cold-induced apoptosis (21, 22). Alternatively, injury to the plasma membrane from extracellular ice recrystallization may cause changes in the cytoplasm, creating stressors for the mitochondria.

A recent study by Tchir et al.(118), demonstrated that mitochondria are very susceptible to freezing injury, and that the membrane viability dye trypan blue was unable to identify cells undergoing loss in mitochondrial integrity following freezing. Previous studies corroborate this finding, demonstrating that the membrane viability

dye, 7AAD, is not able to detect loss of mitochondrial integrity and earlier forms of apoptosis (119). Methods to detect early onset apoptosis include the MTT assay, SYTO 16 probes and, as utilized in this thesis, Annexin-V binding. Tchir et al. further found that the mitochondria, but not the plasma membrane, of single suspension canine kidney cells were highly susceptible to injury between -15°C and -10°C (~ 40% at -15°C to 90% at -10°C for plasma membrane integrity compared to ~ 30% at -15°C to < 5% at -10°C for mitochondrial integrity), which is a similar temperature range for optimal ice recrystallization to occur. It is possible that extracellular ice recrystallization may puncture the cellular membrane only enough to allow for nucleation of intracellular ice crystal formation, but not sufficient to rupture the cell or permanently injure the cell membrane. It is important to note, however, that Tchir et al. did not utilize cryoprotectants for their study, and the mitochondria in the cell types they assessed may have been overly sensitive to freezing conditions.

Nonetheless, these findings, along with studies demonstrating the importance of mitochondrial injury in cryopreservation-associated cell death, suggest that intracellular ice recrystallization injury to mitochondria may be a potentially important mechanism of cryopreservation associated apoptosis. Such a phenomenon would account for the results described in this thesis, which demonstrated a relationship between carbohydrate mediated IRI and improved recovery of nonapoptotic cells. Future studies assessing the role of ice recrystallization and its contribution to mitochondrial damage, along with further identifying the role that mitochondrial injury may have on cell functionality, would provide a more in depth understanding of cryopreservation-

associated cellular injury. The MTT assay is one method that could be utilized to measure mitochondrial activity.

Precise measurement of ice crystal grain size has been described previously as a means of describing ice recrystallization-inhibition. IRI is both an intrinsic property of certain molecules and is dependent on other factors such as concentration. In one report, similar ice grain sizes were observed in solutions of various monosaccharides and disaccharides that suggested an important contribution of the stereochemistry of hydroxyl groups to IRI activity (18). It is important to recognize that the carbohydrate solutions used for the splat cooling assay did not contain cells. More specifically, IRI activity used in the study and reported previously was performed in the absence of cells. The impact of cell concentration or cell type has not been fully addressed and the measurement of mean grain size using the splat cooling assay in the presence of cells has not been developed. In addition, MNCs or $CD34^+$ cell uptake of the individual sugars was not assessed, and so it is possible that not all sugars would exert the same osmotic pressures at the same concentrations. Variations in osmotic pressure could affect dehydration of cells during slow cooling, and so could potentially affect viability of the cells. The sugar trehalose, for example, has been reported to optimally cryopreserve mammliam cells when present on both sides of the cell (120). However, most sugars have been reported to remain predominantly in the extracellular compartment during the cryopreservation of mammalian cells (2, 9, 121), and so it is unlikely that the different sugars used in this study exerted large differences in osmotic pressures. Differences in osmotic pressures and solution tonicity would likely have occurred though, between

solutions containing different sugar concentrations. Salt concentrations were not adjusted for between 20mM, 200mM and 500mM sugar concentrations, and so it is possible that differences in recovery of viable and non-apoptotic cells between the different carbohydrate concentrations was due to differences in solution tonicity and osmotic pressures. Other non-IRI properties associated with the individual sugars may also have contributed to differences in recovery of nonapoptotic and viable cells. For instance, the dissacharides trehalose and sucrose have been reported to provide cryoprotective effects by stabilizing cell membranes through hydrogen bonding with polar groups on the plasma membrane (122). Monosaccharides may not be able to provide these same cryoprotective effects to the same extent as disaccharides. The correlations observed in this thesis, however, between IRI of various carbohydrates and the yield of viable non-apoptotic cells following slow thawing of UCB supports the notion that IRI activity as determined using the splat cooling assay may be relevant in cellular solutions, especially under slow thaw conditions where the correlations were strong.

Carbohydrates are natural cryoprotectants, as evidenced by their protective role in specific frog species that can avoid cell damage in sub-zero temperature (38). Cryopreservation of UCB in carbohydrates alone, however, was found to provide limited cryoprotection. Similar results have been documented in a previous study where cryopreservation of an immortalized hematopoietic cell line in sucrose and trehalose resulted in modest yield of viable cells of approximately 25% (43). Interestingly, however, addition of carbohydrates, along with a 5% solution of DMSO, has provided encouraging results in clinical UCB banking (39, 41). It is likely that carbohydrates

cannot protect against the range of cryopreservation injuries in the way afforded by DMSO. Certain carbohydrates, however, may confer improved ice recrystallization inhibition, accounting for the encouraging results observed when carbohydrates are added in combination with DMSO to cryopreservation solutions. Overall, the results from this study do not provide a basis for reconsidering the standard use of DMSO as a cryoprotectant, but do provide a rationale for pursuing the development of novel carbohydrates with increased IRI activity that could improve the practice of clinical UCB storage. Interestingly, a recent study by Matsumura et al (123) demonstrated that polyampholytes possessing superior IRI properties provide similar cryoprotection efficiencies compared to DMSO for murine L929 and rat mesenchymal stem cells.

The mechanism by which carbohydrates exert their beneficial effect has been unclear and prevented strategic development of novel carbohydrates with enhanced cryoprotective properties. Recently, Ben *et al* (18) have successfully correlated hydration of a carbohydrate to its inherent IRI activity using a “hydration index”. This metric proved to be an accurate predictor for carbohydrate hydration and IRI, based on its stereochemical structure. Further work has shown that hydration of an antifreeze glycoprotein is also important for ice recrystallization-inhibition activity (124). Rational design of novel synthetic carbohydrates and glycoproteins that change stereochemical determinants of water bonding, may therefore prove useful for improving cryopreservation of tissues and cells, including UCB.

UCB is increasingly used as a source of hematopoietic stem cells (HSCs) for hematopoietic stem cell transplantation (HSCT). UCB is especially important in HSCT for pediatric patients. The Centre for International Bone Marrow Transplant Registry reports that 20% of HSCT performed for patients under the age of 20 utilize UCB as the source of cells. In Japan, UCB units account for approximately 50% of unrelated donors used in pediatric HSCT (12). In comparison to the more traditional sources of blood stem cells, bone marrow and peripheral blood, UCB has a number of potential advantages, including reduced incidence of graft versus host disease, greater relative content of primitive hematopoetic progenitors, less stringent human leukocyte antigen (HLA) matching requirements and the units are readily available (125-127). Because of the less stringent HLA matching required for UCB transplantations, it is likely that UCB will be an even more important source for HSCT in regions that have had declining fertility rates, and thus fewer sibling donors that can provide HLA matching bone marrow or peripheral blood. This is demonstrated in a study by Allan *et al.* where it was found that the chance of finding a matching sibling donor for patients in Canada had dropped significantly over the past few years as a result of smaller families, and will likely continue to do so over the next decade, thereby placing a greater demand on alternative sources such as UCB (128). Despite the need for UCB and the advantages associated with it, the application of UCB has limitations in adult patients due to the limited volume, and hence the limited number of cells that can be collected and transplanted (129). For UCB, the dose of total nucleated cells (TNCs) and hematopoietic stem cells infused per kg have been demonstrated to be the most important predictors for successful engraftment after HSCT(130). UCB banking, the

process of storing of UCB units for later use is now common in many countries and allows access to increasing numbers. Cryopreservation of UCB, however, reduces the quantity of functional cells available in UCB units, exacerbating the problem of limited cell doses. Strategies to reduce the loss of functional UCB cells from cryopreservation will be important to increase the application of UCB for hematopoietic stem cell transplantation.

Numerous private and public UCB banks have been established, with most of these banks storing their UCB units by cryopreservation. Although the exact methods of UCB cryopreservation employed by individual banks may vary, a common method consists of freezing at rates of approximately -1 °C/min in a solution containing 5%(v/v) or 10%(v/v) DMSO. While these methods allow for recovery of functional cells post-cryopreservation, a variety of studies have demonstrated that such methods are associated with recovery of increased rates of apoptotic cells and reduced functionality. A study by Yang *et al.* assessing 78 PBSC collections documented that there was a 37% average reduction in the recovery of colony forming unit – granulocyte monocyte (CFU-GM) following cryopreservation, which is another strong predictor of successful hematopoietic stem cell transplantation(131). The majority of studies assessing UCB cryopreservation, however, only report yields of viable cells without quantifying early apoptotic markers. For example, Greco *et al.* found less than 5% of $CD34^+$ cells were non-viable following cryopreservation in DMSO using the cell membrane exclusion dye 7AAD which accumulates in non-viable cells (110). Similar results were found in the standard 5% DMSO conditions used in the present study. However, viability as

measured by 7AAD alone may not be entirely representative of the UCB grafts functionality in the same way that measuring cell viability in cryopreserved autologous PBSC grafts with 7AAD alone did not associate with engraftment rates after HSCT as discussed previously. Other studies have reported levels of apoptosis in the $CD34^+$ progenitor fraction ranging from 20%-70% in cryopreserved UCB units with reduced capacities for CFU formation and homing towards stromal derived factor-1α gradients (56, 57). Stromal derived factor-1α is a common endogenous chemokine for HSC mobilization and homing. Fresh UCB samples can also harbour significant levels of apoptotic $CD34^+$ progenitors depending on the time between collection and analysis. Apoptotic $CD34^+$ progenitors ranged from 24.7% at 6 hours (56) up to 42.28% at 48 hours after collection (132). Reducing apoptosis within the MNC and $CD34^+$ population of umbilical UCB units was considered worthy of study as delayed neutrophil engraftment is commonly observed following UCB transplantation, which is in part due to limited cell number in the small volume collected coupled with the need for cryopreservation (133, 134). Importantly, our results suggest that strategies to reduce cold-induced apoptosis in UCB may allow for more widespread application of UCB, since apoptotic cells may be interfering with the success of HSCT as demonstrated in the second aspect of this thesis. Other methods to overcome the limited cell quantities available in UCB units have been developed, including simultaneous transplantation of two UCB units that are sufficiently matched for their HLA alleles(135, 136), *ex vivo* expansion of $CD34^+$ progenitors(137-139) and intrabone marrow transplantation(140, 141). Even so, reducing cold-induced apoptosis will further minimize apoptotic cells that could interfere with engraftment, and greater understandings of the specific

mechanisms responsible for cold-induced apoptosis are needed to focus future research efforts to optimize cryopreservation procedures.

The results of the second aspect of this thesis suggest that reinfusion of PBSC grafts containing increased levels of apoptotic cells is associated with more prolonged delay of neutrophil recovery after autologous HSCT. Interestingly, study cases with delayed neutrophil recovery received equal numbers of viable mononuclear cells and $CD34^+$ progenitors, in comparison to controls, suggesting that assessment of cell membrane viability alone with 7AAD did not adequately reflect the quantity of functional cells in the grafts. Using Annexin-V to measure earlier forms of apoptosis in multiparameter flow cytometry, the study identified a greater dosage of viable apoptotic MNCs and $CD34^+$ progenitors in PBSC grafts of study cases with delayed neutrophil recovery compared to controls. The number of viable non-apoptotic cells, cells with presumed function intact, was similar between cases and controls, suggesting the possibility that reinfusing apoptotic cells impairs the engraftment potential of functional nonapoptotic hematopoietic stem and progenitor cells within HSC grafts. A study by Zheng *et al.* (142) reported high rates of apoptosis, as measured by annexin-v, in bone marrow $CD4^+$ T lymphocyte population of aplastic anemia patients, in addition to reduced CFU-GM formation when $CD34^+$ stem and progenitor cells were grown in $CD4^+$ T lymphocyte cell culture supernatant from aplastic anemia patients. The group speculated that apoptotic $CD4^+$T cells could inhibit hematopoietic stem and progenitor proliferation by secreting cytokines like tumer necrosis factor-α and interferon- γ that reduce cyclin D3 expression in HSCs required for cell cycle progression. Trebeden-

Negre et al. have also shown that increased quatities of the cytokines; interleukin$_1$-β, interleukin-6, elastase and matrix metallopeptidase 9 in HSC graft supernatant correlate with delayed neutrophil engraftment. The role of apoptosis in the upregulation of these cytokines was not addressed however, in this report (104). Our findings that total CFU and CFU-GM capacities were reduced in parallel with greater apoptosis in MNC and $CD34^+$ cell populations supports the notion that apoptotic cells may impair hematopoietic function of non-apoptotic cells. Future studies identifying possible factors or cytokines that impair CFU capacity would help to determine the validity of this potential mechanism of inhibition.

Another possible explanation of how co-infusing apoptotic cells may impair the engrafting potential of non-apoptotic hematopoietic progenitors centers on reactive oxygen species (ROS). A recent study by Lewandowski *et al.* (143) demonstrated that reactive oxygen species (ROS) are an important promoter of HSC homing to bone marrow during the first few hours following irradiation and HSC transplantation in NOD-SCID mice. The group found a significantly reduced quantity of transplanted HSCs migrating across the endothelial vasculature into the bone marrow of lethally irradiated mice when the generation of extracellular reactive oxygen species (ROS) near endothelial cells in the bone marrow was inhibited 8 hours before irradiation or directly after irradiation by treatment with antioxidants. The group further demonstrated that increased ROS upregulates vascular cell adhesion molecule expression, a protein necessary for recruiting HSCs into the bone marrow, which allowed transplanted HSCs to home specifically to damaged bone marrow. Of particular importance to engraftment

kinetics, the group also demonstrated that inhibition of extracellular ROS in these lethally irradiated mice reduced HSC engraftment for only the first 9 days after transplanation, but eventually reached similar levels of HSC engraftment relative to lethally irradiated mice not treated with antioxidants by 15 days following transplantation. These results demonstrate the importance of ROS in HSC engraftment kinetics, but not overall long-term reconstitution. In parallel with this study, Tesio *et al.*(144) recently demonstrated that ROS's are important mediators for a signalling cascade in $CD34^+$ progenitors that allows the progenitor cells to mobilize from bone marrow into the blood. From these two studies, it is possible to surmise that ROS play a significant role in directing hematopoietic stem cell homing and mobilization. Interestingly, a study by Sasnoor *et al.* (41) demonstrated that cryopreservation of bone marrow MNCs increases the net intracellular ROS in the graft by more than 6 fold relative to fresh grafts. The group further demonstrated that addition of the enzyme catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen, to cryopreserved bone marrow MNCs reduced the net intracellular ROS generation by 2 fold relative to grafts cryopreserved without catalase, which was accompanied by significantly decreased percentages of apoptotic MNCs as measured by Annexin-V in cryopreserved grafts. Other studies have also documented high rates of apoptosis to be associated with increased ROS generation in various cryopreserved mammalian cells (22, 145, 146). As ROS can diffuse across membranes (147), it is possible that increased quantities of transplanted apoptotic cells results in increased ROS released into the blood, which in turn could lead to the upregulation of enzymes that catalyze the decomposition of ROS's. Extracellular glutathione peroxidase and extracellular

superoxide dismutase are two examples of enzymes that can be upregulated in response to increased concentrations of ROS in nearby endothelial cells and macrophages (148-150). In turn, these enzymes may catalyze the decomposition of extracellular ROS's near bone marrow endothelial cells that are important for HSC migration to bone marrow microenvironment. If this hypothetical situation does occur, infusion of large quantities of apoptotic cells may indirectly inhibit homing of nonapoptotic HSCs to bone marrow. Even if antioxidants are not upregulated in the blood upon transplantation of apoptotic cells containing increased ROS, it is possible that ROS associated with apoptotic cells could interefere with homing to appropriate areas of bone marrow by upregulating VCAM-1 expression in endothelial cells in areas that are not damaged and not receptive to engraftment. Overall, apoptotic cells may interfere with homing of hematopoietic stem and progenitor cells into damaged bone marrow, which is an important process to ensure rapid and successful engraftment (151). This proposed mechanism would implicate the heterogenous MNC population and would not necessarily involve any apecialized subset of cells within HSC grafts, and so could produce significant global effects related to ROS's.

Apoptotic $CD34^+$ stem and progenitor cells within HSC grafts may also indirectly contribute to inhibiting engraftment of nonapoptotic $CD34^+$ stem and progenitor cells by competing for space in the bone marrow. Following transplantation, stem and progenitor cells compete for transendothelial migration into bone marrow and subsequent lodging in the marrow microenvironment or stem cell niche(151). Transplanting increasing quantities of apoptotic stem and progenitor cells may increase the likelihood that

apoptotic hematopoietic progenitor cells will migrate to bone marrow, in place of viable nonapoptotic hematopoietic stem and progenitor cells. These apoptotic progenitors would likely be non-functional and would become nonviable soon after. These apoptotic cells may lodge in the bone marrow long enough to interfere with the first few days following transplantation that is a critical time for hematopoietic stem and progenitor cell homing to bone marrow (151). Although Shim et al. have demonstrated that apoptotic hematopoietic $CD34^+$ cells have been demonstrated to possess limited engraftment capacity (56), they did not assess whether this limited engraftment was due to an inability of apoptotic progenitors to home to bone marrow immediatly after transplantation. Since their studies assessed engraftment 8 weeks after transplantation, it is possible that apoptotic progenitors are able to home to bone marrow initially, but die shortly after engrafting.

It is evident then that apoptosis in both MNC and $CD34^+$ populations could inhibit engraftment of nonapoptotic $CD34^+$ progenitors through various mechanisms. However, this study did not address any of those specific mechanisms and so further studies will be required to identify apoptotic mechanisms that could inhibit nonapoptotic HSC engraftment. A widescale proteomic comparison between the blood and bone marrow of NOD-SCID mice receiving fresh or cryopreserved hematopoietic stem cell grafts would aid in identifying possible cytokines and antioxidants that may affect engraftment of functional nonapoptotic hematopoeitic stem cells, in addition to providing insight on other potential mechanisms.

In addition to role of apoptotic cells in PBSC grafts, other factors have been identified that may affect engraftment. A study by Oran *et al.* (152) has identified the extent of exposure to prior alkylator therapy and female gender as two factors that affect neutrophil engraftment. From the same study, preserved renal function and absence of neutropenic fever after transplantation were found to accelerate platelet engraftment specifically (153).

In another study, Ergene *et al.* found that a more prolonged interval between diagnosis and transplantation along with conditioning using the BEAM chemotherapy regimen was associated with delayed neutrophil engraftment in univariate but not multivariate analysis (154). As such, no cause and effect relationship could be determined in their study. Additionally, various studies have shown that progenitor cell surface markers in addition to $CD34^+$ can predict rates of neutrophil and platelet engraftment compared to levels of $CD34^+$ progenitors alone. (155-157) Both total colony and CFU-GM forming capacities have been correlated with engraftment kinetics (158-160) although the methodology continues to have significant inter-laboratory variability and the testing is time consuming and laborious. It is possible that some of these factors contributed to delayed neutrophil recovery in our study. Importantly, however, the cases and controls appeared well balanced in terms of disease severity and other clinical parameters and our study was focused on patients undergoing transplantation for lymphoma, thereby providing a relatively homogenous patient population. Further studies of delayed engraftment assessing the full spectrum of

factors in a prospective manner may then clarify more precisely the role of apoptotic cells in PBSC grafts in relation to other factors.

The impact of cryopreservation on apoptosis in PBSC grafts has also been investigated previously and our results are consistent with the observation that apoptosis occurs to a larger extent in the MNC population compared to the $CD34^+$ fraction. Abrahamsen *et al.* reported mean levels of apoptosis in viable mononuclear cells from cryopreserved PBSC grafts of 32% yet only in 7% of viable $CD34^+$ progenitors (161). Interestingly, more significant levels of apoptosis were reported in PBSCs cryopreserved in 10% DMSO compared to 5% DMSO, reported in a separate study, suggesting that DMSO may contribute to the induction of apoptosis (162). In addition to factors related to cryopreservation, rates of apoptosis in $CD34^+$ progenitors were increased when collected following a mobilization strategy that involved chemotherapy compared with samples obtained following cytokine stimulation (163). The results from our work also suggest a major effect of cryopreservation as apoptosis in both the MNC and $CD34^+$ progenitor populations was minimal in fresh PBSC samples after leukopheresis and processing but more extensive in cryopreserved PBSCs. Other studies have also noted minimal apoptosis (<5%) and viability loss (<5%) in fresh PBSC grafts (161, 163). It appears likely that cryopreservation conditions and thawing procedures contribute most significantly to the induction of apoptosis. In one study, cryopreservation of bone marrow mononuclear cells was shown to induce artificial cleavage of apoptosis related proteins resulting in increased apoptosis of mononuclear cells (164). Overall, the data suggest that cold-induced apoptosis in both the MNC and

$CD34^+$ fractions can reduce hematopoietic stem cell engraftment. Strategies that minimize cold-induced apoptosis, therefore, will be important to ensure safe and efficient HSC transplantion, and may have implications in other areas of cell therapy where cells and tissues may require prolonged storage.

Summary / Conclusion

In summary, the studies described in this thesis suggest that IRI could be an important mechanism for optimizing the cryopreservation of cells, particularly in the storage and banking of UCB, based on the association between carbohydrate IRI activity and the yield of nonapoptotic cells in thawed UCB units. IRI activity of carbohydrates was particularly important for reducing cold-induced apoptosis in both the MNC and $CD34^+$ populations in UCB units. In the second aspect of this thesis, apoptotic MNC and $CD34^+$ cells in thawed blood stem cell products were associated with reduced hematopoietic function in patients undergoing transplantation, potentially through inhibiting the engraftment and functioning of viable and nonapoptotic hematopoietic stem and progenitor cells. Taken as a whole, this thesis provides a basis for further studies on the role of ice recrystallization as an important source of cryopreservation injury and cold-induced apoptosis that may be applicable to several cell types and tissues. Moreover, the results provide a foundation to develop novel carbohydrate-based cryoprotectants possessing strong IRI activity as a means to optimize the storage of cells and tissues.

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Collaborators Contributions

I would like to thank collaborators at the Canadian Blood Services (Michael Halpeny, Lin Yang and Dr. Anthony Giulivi) for providing all aliquots of the peripheral blood stem cell grafts assessed in this study, and for having collected, processed and cryopreserved, and enumerated the cells of these samples. I would like to thank them for providing me with information on the absolute cell numbers of both $CD34^+$ and MNCs that were transplanted into each patient.

I would also like to thank collaborators at the Ottawa Hospital (Dr. James Haebe) for obtaining UCB units and providing them to us. Also, Dr. Ayman Al-hejazi at The Ottawa Hospital identified delayed engraftment cases and case controls in the nested case control study

Finally, I would also like to thank collaborators at the University of Ottawa (Elizabeth von Moos, Jacqueline Tokarew and Jennifer Chaytor) for performing all of the ice recrystallization experiments to obtain sugar IRI ratios and providing me with their results.

Appendices

Appendix A- Supplementary Colony forming unit capacity and colonies infused per kg in delayed engraftment cases vs matched controls

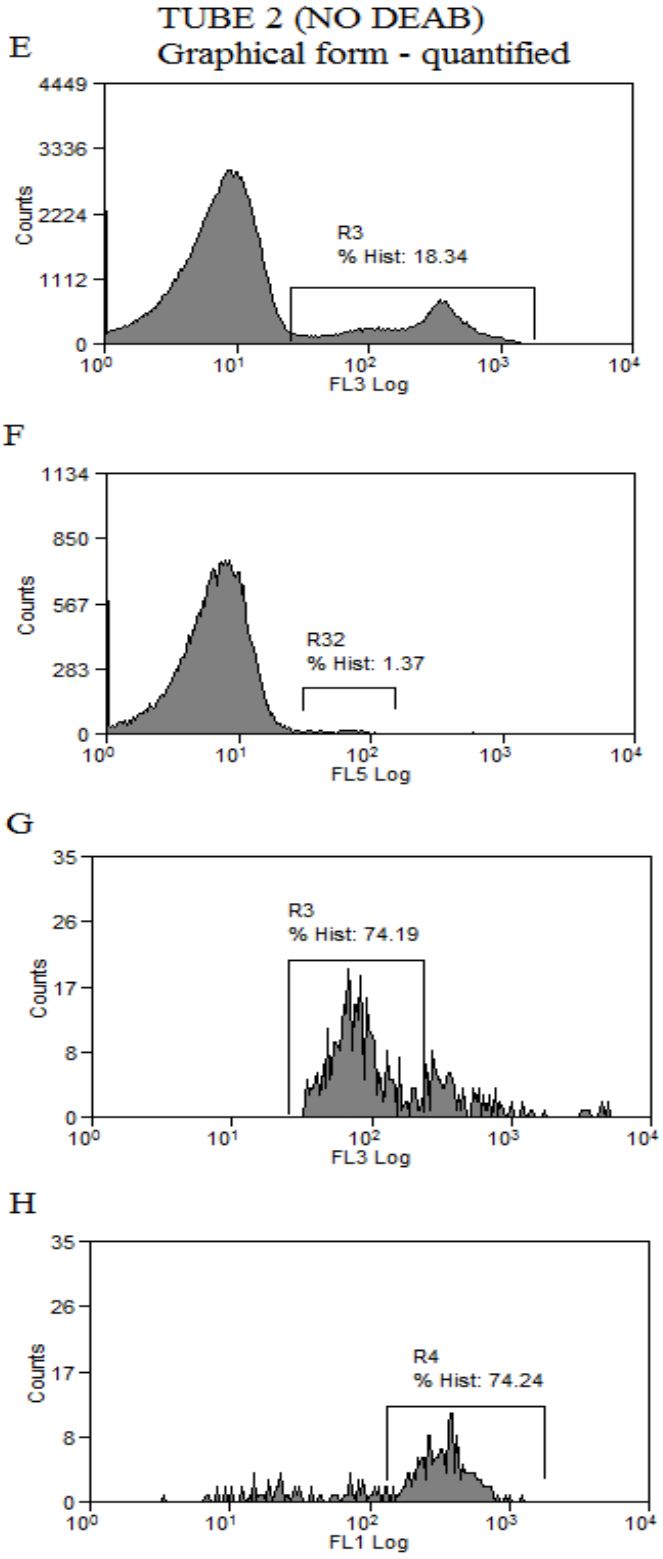
Various CFU capacities per 2 x 10^5 cells between delayed engraftment and matched controls			
	Delayed engraftment	Matched controls	p
CFU-GEMM	0.64 ± 0.23	1.29 ± 0.37	0.22
BFU-E	9.15 ± 1.64	26.52 ± 5.75	0.03
CFU-E	5.47 ± 0.87	7.89 ± 1.56	0.27

The plating efficiency of the various CFU colonies. BFU-E are decreased in PBSCs of study cases with delayed neutrophil engraftment in comparison with controls ($p<0.05$). CFU-GEMM and CFU-E plating efficiencies are not significantly ($p>0.05$) different between delayed engraftment study cases and matched controls.

Various CFU infused / kg between delayed engraftment and matched controls			
	Delayed engraftment	Matched controls	p
CFU-GEMM	$3.60 \times 10^3 \pm 1.3 \times 10^3$	$6.30 \times 10^3 \pm 2.0 \times 10^3$	0.34
BFU-E	$5.46 \times 10^4 \pm 0.90 \times 10^4$	$1.31 \times 10^5 \pm 0.26 \times 10^5$	0.04
CFU-E	$3.34 \times 10^4 \pm 0.48 \times 10^4$	$4.34 \times 10^4 \pm 0.90 \times 10^4$	0.42

The various colonies infused per kg. BFU-E / kg are decreased in PBSCs of study cases with delayed neutrophil engraftment in comparison with controls ($p<0.05$). CFU-GEMM and CFU-E / kg are not significantly ($p>0.05$) different between delayed engraftment study cases and matched controls.

Appendix B - Supplementary Graph form of gating strategy used in PBSC flow cytometry analysis



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1. **Wu LK**, Tokarew JM, Chaytor JL, von Moos E, Li Y, Palii C, Ben RN, Allan DS. Carbohydrate-mediated inhibition of ice recrystallization in cryopreserved human umbilical cord blood. Carbohydrate Res, 2010.

Manuscripts submitted for peer review:

1. **Wu L**, Al-Hejazi A, Filion L, Ben R, Halpenny M, Yang L, Giulivi A, Allan DS. Increased apoptosis in cryopreserved PBSCs and delayed neutrophil recovery after transplantation: a nested case-control study. Cytotherapy (submitted Nov 20 2010, resubmitted May 19th, 2011)

2. **Wu L**, Martin T, Li Y, Yang L, Halpenny M, Giulivi A, Allan DS. Cell aggregation in thawed hematopoietic stem cell products visualized using micro-flow imaging. Transfusion Medicine (Submitted as a short communication May 2011).

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4. Leclère M, Kwok BK, **Wu LK**, Allan DS, Ben RN. Synthesis of C-linked AFGP analogues and assessment of their cryoprotective properties. Org Lett (submitted October 5, 2010)

Other

1. **Luke Wu,** Robert Ben, David Allan Effects of an antifreeze glycoprotein analogue on cryopreservation of umbilical cord blood units (Dissertation, Accepted April 2008) University of Ottawa, Dept of Biochemistry, BSc thesis.

Presentations:

The following list includes oral presentations for which I presented:

1. *10th Annual OHRI Research Day* (November 18th 2010) **Luke Wu,** Ayman Al-Hejazi, Lionel Filion, Robert Ben, Michael Halpenny, Lin Yang, Antonio Giulivi, David S. Allan Increased apoptosis in cryopreserved autologous PBSCs and delayed neutrophil recovery after transplantation: a nested case-control study

2. *Third annual Hematology Update - The Ottawa Hospital* (October 28th 2010) **Luke K. Wu**, Ayman Al-Hejazi, Lionel Filion, Robert Ben, Michael Halpenny, Antonio Giulivi, David S. Allan Increased apoptosis in cryopreserved autologous PBSCs and delayed neutrophil recovery after transplantation: a nested case-control study

3. *16th Annual International Society of Cell Therapy meeting* (May 25th 2010) **Luke Wu,** Ayman Al-Hejazi, Lionel Filion, Robert Ben, Michael Halpenny, Lin Yang, Antonio Giulivi, David S. Allan Apoptosis in cryopreserved autologous PBSCs and delayed engraftment after transplantation despite sufficient CD34(+) cells

4. *BMI Research Symposium,* (February 17th 2010) **Luke Wu,** Ayman Al-Hejazi, Lionel Filion, Robert Ben, Michael Halpenny, David S. Allan. Improving Umbilical Cord Blood Cryopreservation By Reduction Of Cryopreservation-Induced Apoptosis

The following list includes poster presentations for which I presented:

1. *10th Annual OHRI Research Day* (November 18th 2010) **Luke Wu,** Teresa Martin, Michael Halpenny, Antonio Giulivi, David Allan. Cell aggregation in thawed peripheral blood stem cell products visualized using micro-flow imaging

2. *9th Annual OHRI Research Day* (November 27th 2009). **Luke Wu**, Jackie Tokarew, Elizabeth Von moos, Robert Ben, David Allan. Ice Recrystallization Injury and Cryopreservation Damage in Umbilical Cord Blood Units

3. *2nd Annual Hematology Update* (October 8th 2009) **Luke Wu**, Jackie Tokarew, Robert Ben, David Allan. Ice Recrystallization Injury and Cryopreservation Damage in Umbilical Cord Blood Units

4. *University of Ottawa Graduate Biochemistry Poster Day* (May 21st 2009) **Luke Wu**, Jackie Tokarew, Elizabeth Von moos, Robert Ben, David Allan. Ice recrystallization as a contributing mechanism to cryopreservation induced damage in cord blood units

5. *University of Ottawa Undergraduate Biochemistry Poster Day* (April 8th 2008) **Luke Wu**, Elizabeth Von moos, Robert Ben, David Allan. Effects of an antifreeze glycoprotein analogue on cryopreservation of umbilical cord blood units.

The following list includes presentation I was involved in but did not present:

1. **Poster Presentation** *52nd ASH Annual Meeting and Exposition* (December 5th 2010) **Luke Wu,** Teresa Martin, Michael Halpenny, Antonio Giulivi, and David S. Allan. Analysis of Cell Aggregation In Thawed Hematopoietic Stem Cell Products Using Micro-Flow Imaging

Published Abstracts:

The following list includes the published abstracts I presented

1. **Wu L,** Al-Hejazi A, Filion L, Ben R, Halpenny M, Giulivi A, Allan DS. Apoptosis in cryopreserved autologous PBSCs and delayed engraftment after transplantation despite sufficient CD34(+) cells. Cytotherapy, 2010. (in press) [oral]

The following list includes published abstracts I was involved in but did not present:

2. **Wu LK,** Martin T, Halpenny M, Giulivi A, Allan DS. Analysis of cell aggregation in thawed hematopoietic stem cell products using micro-flow imaging. Blood, 2010 (in press) [poster].

3. **Wu L,** Tokarew J, Chaytor J, von Moos E, Li Y, Palii C, Ben R, Allan D. Carbohydrate-mediated inhibition of ice recrystallization in cryopreserved human umbilical cord blood. Bone Marrow Transplantation, 2010;45:S156-7 (P590). [poster]

Extra-curricular activities:

Board member and cohost for University of Ottawa science radio show "Peer Review Radio" (May 2010 – Ongoing)

- Directed and produced 6 shows which can be found on our website at www.peerreviewradio.com

Let's Talk Science (May 2009 – Ongoing)

- Learned to effectively mentor and teach colleagues and students.
- Taught a lecture to grade 12 students concerning genetics and DNA isolation

Distress Centre – Mental Health Crisis Line volunteer (October 2008 – ongoing)

- Assisted those in a distressful/crisis situation through self-help and awareness of community resources.

Operating room escort at CHEO (December 2008 - Ongoing)

- Entertained children and help relieve their (and the parents) stress prior to their operation.
- Assisted the medical team with any needs prior to anesthesia of the patient.

Board member and co-founder of Scientific Innovation for International Development (September 2008 –September 2009)

- Organized presentations and campaigns through designating task leaders and overseeing the completion of the task.
- Fulfilled necessary administrative work to create the club under the student federation

Co-founder Students Biochemistry curriculum committee (September 2007 - May 2008)

- Along with 4 other students and the chair for the BMI curriculum for the University of Ottawa, we founded the SBCC to enhance student representation on curriculum matters.