The impact of cryopreservation on the function of hematopoietic stem and progenitor cells

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ABSTRACT

Cryopreservation is currently the only method allowing for the long-term preservation of hematopoietic stem and progenitor cell (HSPC) grafts until their use. However, cryoinjuries reduce cell viability and potency of HSPC. New cryoprotectant (CPA) solutions have recently emerged that have not yet been investigated that may improve the cryopreservation of HSPCs. The overarching hypothesis of the work described in this thesis, is that different CPAs have diverse impact on the key biochemical processes essential for HSPC homeostasis which influences post thaw cell viability and potency. To test this hypothesis, 4 CPAs were extensively characterized for their cryoprotective properties on cord blood (CB) HSPCs in comparison to DMSO control. CryoProtectPure (CPP) supported similar post thaw cell viability and engraftment as DMSO control, whereas pentaisomaltose (PIM) and cryonovo (CN) failed as CPAs for HSPCs. Subsequently, the impact of CPAs on key biological pathways was explored to identify potential biochemical pathways implicated in HSPC cryopreservation. The impact of CPAs on cell membrane integrity, oxidative phosphorylation, glycolysis, and autophagy was examined. CPP and DMSO had varying impact on glycolytic and mitochondrial respiratory activities of HSPCs post-thaw, whereas both CPAs as well as PIM and CN had negligible impact on cell membrane parameters prefreeze. Cryopreservation and thawing strongly induced autophagy in HSPCs. Importantly, early inhibition of autophagy with 3-Methyladenine (3-MA) reduced the recovery of functional CB HSPCs post thaw. Together, my findings provide new insights regarding the biological processes impacted by CPAs and cryopreservation of HSPCs and identify potential targets to improve cryopreservation of HSC grafts.

This dissertation is dedicated to my family for their magnanimous love showered on me and having persistent confidence in all my endeavours.

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to do hard work and remain focussed in my efforts with their profound faith in me, despite of their own work pressures and commitments. This thesis is as much yours as it is mine.

DECLARATION

I, Richa Kaushal, hereby declare that the work presented in this thesis is my own research work. Wherever contributions of others are involved, every effort has been made to indicate that clearly, with due reference to the literature, and acknowledgement of collaborative research.

Richa Kaushal

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Table of Contents

List of figures

List of tables

ABBREVIATIONS

3-MA	3- methyladenine
ALDH	Aldehyde dehydrogenase assay
AMPK	AMP- activated protein kinase
ANOVA	Analysis of variance
ATG	Autophagy related genes
ATP	Adenosine triphosphate
BFU-E	Burst forming unit- erythroid
BM	Bone marrow
BSA	Bovine serum albumin
CB	Cord blood
CBT	Cord blood transplantation
CBU	Cord blood unit
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU-GEMM	Colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM	Colony forming unit- granulocyte, macrophage
CFU	Colony forming unit
CIHR	Canadian Institutes of Health and Research
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CN	CryoNovo
CPA	Cryoprotectant or cryoprotective agent
CPP-STEM	CryoProtectPure
CSL	CryoScarLess
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleotide
DMSO	Dimethyl sulfoxide
ECAR	Extracellular acidification flux Rate

EG	Ethylene glycol
EPC	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
EryP	Erythroid progenitor
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FACT	Foundation for the Accreditation of Cellular Therapy
FITC	Fluorescein isothiocyanate
FLT-3	Fms-related tyrosine kinase 3
G-CSF	Granulocyte - colony stimulating factor
GM	Granulocyte macrophage
GMP	Granulocyte monocyte progenitor
GVHD	Graft versus host disease
HES	Hydroxyethyl starch
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	HSC transplantation
HSPC	Hematopoietic stem and progenitor cell
IIF	Intracellular ice formation
IL-3pSTAT5	Interleukin 3 Phosphorylated transducer and activator of transcription 5
IRI	Ice recrystallization inhibitor
ISHAGE	International Society of Hematotherapy and Graft Engineering
JAK/STAT	Janus kinase/signal transducers and activators of transcription
LDH	Lactate Dehydrogenase Assay
LP	Lipid Peroxidation
LT-HSCs	Long term- HSCs
LTC-IC	Long term culture-initiating cell
MEP	Megakaryocyte erythroid progenitors
MNC	Mono nuclear cell
MPP	Multipotent progenitor

MSC	Mesenchymal stem/stromal cell
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NOD	Nonobese diabetic
NSG	NOD SCID gamma mouse
OCR	Oxygen Consumption Rate
OXHOS	Oxidative Phosphorylation
PBS	Phosphate buffered saline
PB	Peripheral blood
PBSC	Peripheral blood stem cell
PE	Phycoerythrin
PEG	Polyethylene glycol
PEP	Platelet engrafting progenitor
PG	Propylene glycol
PI3K	Phosphoinositide 3-kinase
PIM	Pentaisomaltose
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SCF	Stem Cell Factor
SCID	Severe combined immune deficient
SD	Standard deviation
SEM	Standard error of the mean
SR 1	StemRegenin 1
SRC	SCID repopulating cells
STAT3	Signal transducer and activator of transcription 3
ST-HSCs	Short term-HSCs
TCA	Tricarboxylic acid

TPO	Thrombopoietin
TNC	Total nucleated cell
TNF	Tumor necrosis factor
UCB	Umbilical cord blood
XF	Extracellular flux

CHAPTER 1

INTRODUCTION

1.1. Hematopoiesis and hematopoietic stem cell (HSCs)

1.1.1. Hematopoiesis

Hematopoiesis is a continuous process of formation and development of various types of blood cells with divergent functions such as red blood cells, platelets, neutrophils, and lymphoid cells to name a few[1]. In a healthy adult human, roughly 10 billion to 100 billion new blood cells are produced per day, to maintain steady state levels in the peripheral circulation. This means that over a lifetime, an average human may produce about 2.9 quadrillion to 29 quadrillion new blood cells from HSCs[2]. These adult blood cells are constantly regenerated throughout the life from HSC through a series of lineage committed stem cells differentiating into various populations of progenitors. The number of adult blood cells that are produced from HSCs depends on the body's needs and the lifespan of each type of blood cell[3].

Bone marrow (BM) is the primary site of generation of blood cells in humans. Each type of blood cell has a different developmental pathway and a different set of markers that can be used to identify them[4]. For example, red blood cells develop from HSCs through erythroid progenitors and normoblasts to reticulocytes and finally erythrocytes. White blood cells can be divided into three broad groups: granulocytes (such as neutrophils, eosinophils, and basophils), lymphocytes (such as T cells, B cells, and natural killer cells), and monocytes (which can differentiate into macrophages or dendritic cells)[5]. Each group has its own lineage and differentiation stages. Platelets are formed from terminal maturation of megakaryocytes, which are large cells in the bone marrow that undergo multiple rounds of deoxyribonucleic acid (DNA) replication without cell division[6].

1.1.2. Hematopoietic stem cells and progenitors (HSPCs)

HSCs and all stem cells are functionally defined by two key properties - self-renewal and multilineage differentiation[7]. Besides these characteristics stem cells possess other properties like being mostly quiescent and very rare[6]. Indeed, stem cells exist in small numbers in specific locations called niches[8]. The niches provide a supportive environment for HSC to maintain their quiescence and potency[9]. Stem cells are mostly in a state of quiescence, which means they do not divide or differentiate unless they receive signals from their niche or from external stimuli[10]. Quiescence allows stem cells to preserve their genomic integrity, avoid senescence, and regulate their population size [11]. Conversely, apoptosis is important for maintaining tissue homeostasis and preventing tumorigenesis. Stem cells can be induced to undergo apoptosis by various factors, such as DNA damage, oxidative stress, inflammation, or loss of niche signals[12]. Apoptosis can also regulate the balance between self-renewal and differentiation of stem cells[13].

HSCs are not a homogeneous population, but rather consist of different subsets that have different functional properties. One way to classify HSCs is based on their engraftment potential, which refers to their ability to reconstitute the hematopoietic system of a recipient after transplantation and the length of engraftment [14]. Long-term engraftment HSCs (LT-HSCs) are the most primitive and rare subset of HSCs. They have the capacity to sustain hematopoiesis for the lifetime of the recipient and can give rise to all the major blood cell lineages[15]. LT-HSCs are mostly quiescent, and do not divide or differentiate unless they receive signals from their niche or from external stimuli[16]. LT-HSCs also have high expression of genes involved in DNA repair, antioxidant defense, and stem cell maintenance[2].

On the other hand, short-term engraftment HSCs (ST-HSCs), also called multipotent progenitors (MPPs), are a more differentiated and abundant subset of HSCs.

They have the capacity to sustain hematopoiesis for weeks to several months, but not for the lifetime of the recipient. They can give rise to most, but not all, blood cell lineages[17]. ST-HSCs are more proliferative than LT-HSCs, so they divide and differentiate more frequently. Proliferation and differentiation allow ST-HSCs to rapidly produce mature blood cells in response to hematopoietic demand[18].

The functional properties of self-renewal that differ between LT-HSCs and ST-HSCs are influenced by various factors, such as transcriptional, post-transcriptional, and epigenetic regulators, signaling molecules, metabolic pathways, cellular stress responses, and niche interactions[19]. For example, some of the genes that have been shown to regulate self-renewal in LT-HSCs are *Bmi1*, *Runx1*, and *Tel2*. Different genes that regulate self-renewal in ST-HSCs are *C/EBPα, Egr1, Gata2* and *PU*12[15]. The signaling pathways shown to regulate self-renewal in both LT-HSCs and ST-HSCs are Notch, TGF-β, and Janus kinase/signal transducers and activators of transcription (JAK/STAT)3[20].

Decades of experimentation with various xenotransplant mice models has enabled scientists to further enrich our knowledge of HSC and hematopoiesis has been defined as a hierarchal model supported by self-renewing HSCs that belong to the apex of its pyramidical form (Figure 1)[21]. In all established models of hematopoiesis, HSCs undergo long-term self-renewal while further giving rise to all the blood lineages. The classical model claims that the self-renewing function of HSC is gradually lost as they transfer into MPP states which eventually give rise to progenitor cells that begin the myeloid and lymphoid branches[22]. All the common lineages for myelopoiesis (common myeloid progenitor - CMP) and lymphopoiesis (common lymphoid progenitor - CLP) are derivatives of these MPPs. The oligopotent CMPs undergo further restriction into bivalent Granulocyte monocyte progenitors (GMPs) and megakaryocyte erythroid progenitors (MEP) (Figure 1A, Figure 1B)[21].

However, over the years, hematopoietic models have evolved. Various independent investigations using bulk and single cell ribonucleic acid (RNA)-sequencing technologies have demonstrated the developmental structure of HSC, as it gives rise to a sequence of progenitor cell intermediates which further undergo a continuing fate restriction[21]. A complicated roadmap endures in the lineage relationships between stem cells, progenitors and mature cells throughout the developmental transitions centered on molecular basis. Woolthuis, C.M., *et al.*[5]reported that heterogeneity within the HSC population is related to lineage potential, including a megakaryocyte-biased HSC that directly gives rise to megakaryocyte progenitors and bypasses classical intermediate commitment stages, including the CMP and MEP (Figure 1C).

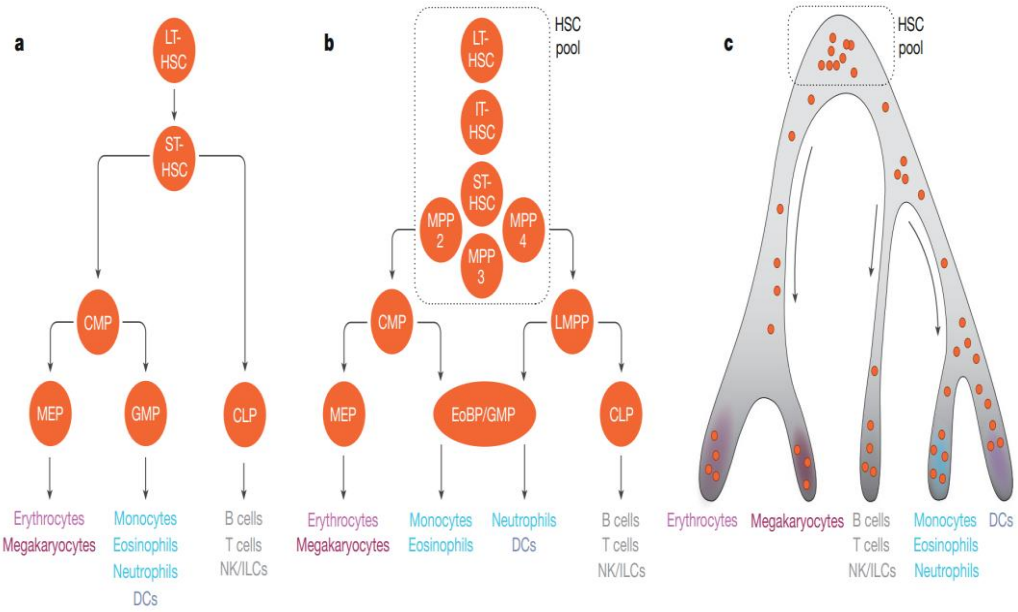

Figure 1: Model of hematopoietic hierarchy.

(a) Classical model of the hematopoietic hierarchy with a strict separation between the myeloid and lymphoid branches as the first step in lineage commitment downstream of the HSC. (b) New findings showed that the HSC pool is now accepted to be more heterogeneous both in terms of self-renewal and differentiation properties, the myeloid and lymphoid branches remain associated further down in the hierarchy via the lymphoid-primed multipotential progenitor (LMPP) population. (c) From 2016 onwards, single-cell transcriptomic snapshots indicate a continuum of differentiation. Each red dot represents a

single cell and its localization along a differentiation trajectory. Figure adapted from E Laurenti & B Göttgens, 2018[6]

Notta, *et al.* showed a hierarchy primarily made of two-tiers in adult HSCs. The top-tier comprises multipotent cells such as HSCs and MPPs and a bottom-tier is comprised of committed unipotent progenitors. They have also stated that, the origins of the megakaryocyte lineage branch change from human fetal liver to adult BM. Also in fetal liver, enrichment of megakaryocyte progenitors is not restricted to the stem cell compartment whereas in BM, the fate of multipotent cells is strongly tied to Mk lineage (Figure 2)[11].

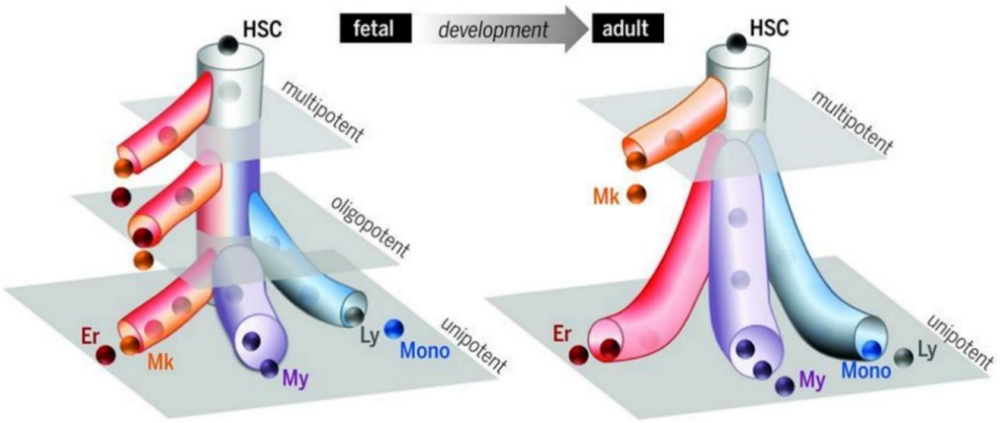

Figure 2: Refined model of human blood development.

The redefined model envisions a developmental shift in the progenitor cell architecture resulting in a two-tier hierarchy by adulthood. Figure adapted from Notta, *et al.* 2016[11]

1.1.3. Markers for human HSCs

One of the main challenges for researchers is the detection and separation of HSC from the larger pool of non-HSC that includes mature cells and committed progenitors. It is estimated that HSC represents approximately 1 in 10,000-15,000 cells of the BM and 1 in 100,000 nucleated cells in the blood[23]. Identification of HSC population is challenging as they are rare and do not express a unique cell surface marker, so not a single antigen can identify HSC[11] (Figure 3).

HSC can be differentiated from differentiating progenitors and mature blood cells by the lack of expression of surface markers normally present on mature hematopoietic cells such as CD19 and CD3 for B- and T-cells, respectively. Hence, "lineage-negative population" are largely depleted of mature cells[8]. Lineage negative cells are however only slightly enriched in stem cells, but lineage depletion is a useful step towards HSC purification either through fluorescent activated cell sorting (FACS) or magnetic isolation using cell surface antigen discussed below.

One of the best markers for HSPCs is CD34. It is a transmembrane phosphoglycoprotein [13], which is highly expressed on most human HSPCs, but not present on mature blood cells. It is however also expressed by endothelial cells and endothelial progenitor cells [24]. Expression of CD34 on the cell surface of HSPCs and its gradual down regulation on more mature cells imply that it can play an important role in the preservation of the undifferentiated HSPCs[25]. Additionally, CD34 has also been hypothesized to play a role in improving cell proliferation [26]. While human HSCs are enriched in CD34+ cells, the use of multiple surface antigen is best to further enrich HSC. Fresh human HSCs are thus enriched in Lin-CD34+CD38- population of cord blood (CB) and BM[1327]. CD38 marker is a protein that is found on the surface of many types of immune cells, such as T cells, B cells, and natural killer cells[28]. It is involved in various functions, such as cell activation, signal transduction, calcium signaling, and cell adhesion[10].

Another marker that used to identify HSCs is CD90[29]. CD90 is expressed by a subset of CD34+ cells that have high repopulating potential and multilineage differentiation capacity[24]. While human HSCs are mostly CD90+, mouse HSCs are mostly CD90lo or CD90- [29]. Baum, *et al.* discovered a rare set of CD90/Thy-1^{+}CD34^{+} human fetal BM cells that is extremely enriched for pluripotent progenitor activities (myeloerythroid, B-lymphoid and T-lymphoid system) and long-term culture initiating ability[30]. Moreover,

Majeti *el al.,* ultimately fractionated the multilineage compartment and long-term HSC by the combined use of the CD90 and CD45RA antigens; long-term HSC were identified by the profile Lin^{-} $CD34^{+}$ $CD38^{-}$ $CD90^{+}$ $CD45RA^{-}$[31]. Finally, Notta, *et al.* reported that HSCs are additionally enriched by the expression of CD49f (~1 in 10 of CD34+ CD38- are HSCs) and identification of MPPs can be done by observing the loss of CD49f expression (Figure 3)[28].

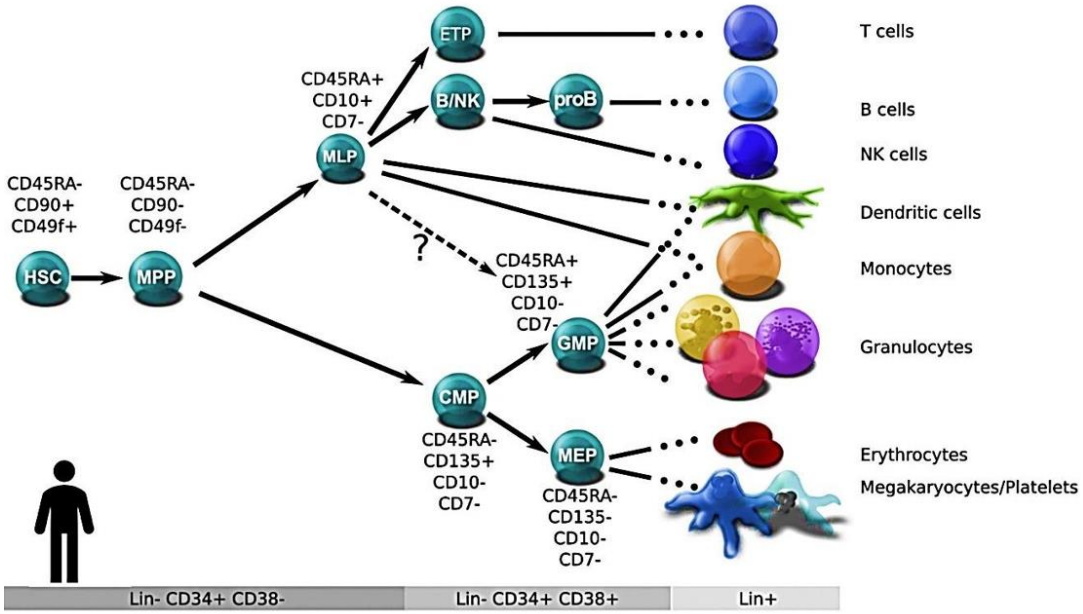

Figure 3: Lineage determination in the adult human hematopoietic hierarchy.

The major classes of stem and progenitor cells are defined by cell surface phenotypes, which are listed next to each population and in the grey bars below each schematic. Figure adapted from Doulatov S., *et al.*[8].

Recently, Fares, *et al.* demonstrated that Endothelial protein C receptor (EPCR/CD201) is a reliable marker in uncultured CB cells and particularly in CB CD34+ cells expanded with UM171 (a pyrimidoindole derivative previously shown to expand CB HSCs)[15]. Also, a study conducted by Caryn Ito *et al.,* demonstrated that $AC133^{+}CD38^{-}$ poses as an enhanced marker that can enable superior tracking and enrichment for Scid repopulating cells (SRC) and long-term culture- initiating cells (LTC-IC) *ex vivo* cultures [33].

However, none of these markers are perfect and there is still no way to isolate purify HSCs at 100% purity. This is an active area of research that aims to further improve our understanding of HSC biology[7,8]. An alternative way of enriching HSC has been based on their high dye efflux capacity, in so-called side population (example-Hoechst, rhodamine)[28,29]. In my thesis, I have tracked HSPCs mainly using the CD34 antigen and combined CD34 with additional antigens such as CD45RA, CD49f, CD90 and EPCR to better measure the impact of cryopreservation and CPAs on HSC.

1.1.4. HSC transplantation (HSCT) - a curative therapy

In 1963, E.D. Thomas was the first person to introduce BM as the foremost source of HSCT[34]. Since then, several improvements in clinical practices have been integrated to improve outcomes for patients[35]. HSCT has become a curative treatment option to treat various blood related diseases and deficiencies. Specifically, it allows for the restoration of the stem cells when the BM gets damaged by harmful radiation or chemotherapy or diseases like leukemia[9]. Each year almost 45,000–50,000 HSCTs are performed around the world for the treatment of leukemia, lymphoma, immune deficiency ailments and for other blood related diseases[36].

HSCT can be categorized into two types -allogeneic and autologous. The main difference between autologous and allogeneic transplants is the source of the stem cells[37]. In an autologous transplant, the stem cells come from the patient's own body while in an allogeneic transplant, the stem cells come from a donor who has a compatible human leukocyte antigen (HLA) type with the patient[38]. HLA proteins or markers are observed on most of the cells, and the immune system makes use of these markers to recognize which cells belong to the host and which do not. Hence, HLA typing is used to find and match donors for patients in allogeneic transplantation. HLA matching is of vital importance for the patient and success outcomes since HLA mismatch has been linked to graft failure,

Graft versus host disease (GVHD) and mortality[9]. In both auto- and allogeneic HSCT, HSC grafts may be cryopreserved when the grafts need to be preserved for significant periods of time.

1.1.5. Principal sources of HSCs

HSC products can be derived from mobilized peripheral blood, BM, and CB. Selection of HSC source relies on factors like transplantation indication and donor accessibility[39]. Currently, mobilized peripheral blood stem cells (PBSCs) are the most common sources of HSCs[40]. PBSCs transplantation is associated with more rapid engraftment in comparison to BM and CB-derived HSCT. The HSC-containing cell product is volume-reduced and frozen until the patient is ready for transplantation[31]. In the case of PBSC, the number of leukapheresis procedures can be decreased to one or two times by mobilization of HSCs to the peripheral blood with granulocyte-colony stimulating factor (G-CSF, 6 μg/kg/day) treatment[2]. G-CSF treatment is a type of drug that helps the BM to make more white blood cells, neutrophils, which are important for fighting infections. Stimulation of healthy allogeneic donors with G-CSF treatment rarely causes adverse events[41], although rare occurrences of nontraumatic splenic rupture and other serious adverse events have been reported[42] .

Lastly, the third conventional source of HSC is umbilical CB, which is the principal source of HSC used in the research described herein. CB unit (CBU), which traditionally was deemed a waste and simply discarded, has become an essential source of HSC for allogeneic HSCT when HLA-matched sibling and unrelated donors are unavailable[43] .

1.1.6. Cord blood transplantation (CBT)

In cord blood transplantation (CBT), HSPCs are derived from the umbilical cord and placenta of a newborn baby[20,44,]. In 1974, it was shown that the cells of CB[15] possessed hematopoietic progenitors[45]. By 1983, the concept of using CB as an alternative source of

HSC for transplant had been proposed[46]. The first successful CB transplantation was performed on a 5-year-old boy with Fanconi's anemia in 1988[47].

In line with this, Wang, J.C., *et al.* reported through limiting dilution transplantation analysis that CB has a higher frequency of HSC than BM or PBSC[18]. Since, CB has become an important source of HSC for allogeneic HSCT when HLA-matched sibling and unrelated donors are unavailable. CB cells are relatively immunologically naive, which allows for multiple-antigen mismatches, though engraftment and the survival outcomes positively correlate with HLA matching[48]. Usually, CB should meet a match of at least 4/6 HLA matching for HLA-A, -B at the antigen level, and HLA-DRB1 at the allelic level[49]. The influence of HLA disparities on the outcome of CBU transplantation is controversial and Gluckman, *et al.* showed that the higher the number of cells, the lower the number of HLA disparities, which is good for better engraftment but the higher the number of HLA disparities, the higher the chance of acute GVHD[19]. Besides Sobol *et al.,* reported that $CD34^+$ cell dose ($\geq 1.5 \times 10^5$/kg) helps to predict faster engraftment and can be useful for graft selection[50]. However, the success of CB HSC transplant depends on the cell dose [51] and one of the main challenges of CB bank is to conserve high cell yields during processing to ensure a high rate of usage of cryopreserved CBUs[52].

According to the World Marrow Donor Association, the total number of CBT performed worldwide up until 2018 was 40,135. This number includes both related and unrelated CBT for various diseases[53]. However, the number of CBT has been declining in recent years, due to several factors, such as the development of haplo-identical HSCT, the limited cell dose of CBUs, and the high cost of cord blood banking and transplantation. Indeed, the development of haplo-transplants has gained significant interest with the introduction of new GVHD prevention strategies and high $CD34^+$ doses to overcome risk of graft failure[53]. A haploidentical donor usually matches 50% of the recipient's HLA and

donor may be the recipient's parent, sibling, or child[54,55]. Also, haplo-cord transplant reported faster immune reconstitution with rapid B-cell and delayed T-cell recovery[56]. However, CB transplantation remains a potentially curative therapy for patients with leukemia, lymphoma, myeloma, and myeloproliferative disorders[37].

CB transplantation possesses several advantages over other types of stem cell sources such as BM or PBSC[57]. One advantage is that it can be easily extracted, resulting in no risk or pain for either the mother or the baby. The includes advantages of CB HSC include the rapid ease of procurement (few weeks), reduced stringent requirements for HLA matching, reduced GVHD disease compared to other stem cell sources, and improved HLA diversity especially important for ethnic population[58].

There are however disadvantages also when it comes to CBT. The principal inconvenience is the longer time for CB HSC to engraft which is associated with high risk of infection in patient[38]. Indeed, prolonged periods of neutropenia, thrombocytopenia and slow recovery of the immune system are the main issues associated with CB transplants[59]. This is partially because cell dose in CBU graft is significantly lower than adult grafts. The platelet engraftment usually occurs a month longer and 1-2 weeks longer for neutrophil in CBT over adult based HSCT (PBSC or BM)[60,61.] In addition, CBU might not contain enough cells to engraft, and the success of CB engraftment is related to CB banking processes[62]. Approximately 10–20% of patients receiving an unrelated CB transplantation fail to engraft[60,63].

Selection of CBUs with sufficient TNC number is challenging for adult patients and a single CBU transplantation is usually not sufficient to provide enough cells[64]. Double CBU transplantation is required for adults to get the minimum threshold of cells required for the procedure though the slow engraftment remains an issue. Moreover, double CBU transplantation did not show any advantage over single CBU transplantation as similar

outcome was observed in both single and double CBU transplantation[65]. Also, delayed platelet recovery and higher GVHD risk were reported after double unit CB transplantation [43]. The reconstitution ability of CB cells might be lost during different stages of collection, handling, processing, manufacturing, storage, and shipment. Other disadvantages include the high cost of the grafts (about US$50,000) and longer hospitalization stays[66] .

In summary, CBT is a valuable option for patients who need HSCT but lack a suitable donor or have a high risk of relapse. In the near future, CBT may also benefit from recent developments in cord blood [67]expansion technologies and other strategies to improve engraftment and immune reconstitution post-CBT[44,53,68]. Another option would also be to improve the outcomes of freezing and thawing on the viability and potency of CB HSPCs through improved cryopreservation. The latter could be achieved by identifying superior CPA solutions and by identifying biochemical avenues to improve HSPC potency post-thaw. For these reasons, I investigated in my thesis new CPAs and the impact of cryopreservation and/or these CPAs on key biochemical pathways important for post-thaw cell survival and potency.

1.2. CBU banking and assays used to measure graft quality and potency

Public banking of CB was initiated in 1992 at the New York Blood Center (New York, NY, USA)[69]. CB banking is related to processing, testing, cryopreservation, storage, and distribution of CBUs intended for HSCT. In Canada, Canadian Blood Services began a national public CB bank in 2013 with an aim to store high quality of CBUs to meet the increasing demand[70]. Assessment of graft adequacy is crucial before a CBU can be banked and used for transplantation. Various factors associated with collection procedures, donor dependence, transportation etc. can impact the yield and quality of CBU product[69]. Most of the CB banks follow the international standards for CB collection, banking, and release. The NETCord and the Foundation for the Accreditation of Cellular Therapy (FACT)

together provide standards regarding collection, processing, and storage. NetCord is an association of CB banks founded in 1997 to promote the highest quality in CB products and to encourage and facilitate the use of CB transplants[71]. The NetCORD-FACT publishes a series of standards designed to ensure high quality of CBU across all public CB banks. These provide guidelines to support the procedures related to CB management, collection, and processing to name a few[71]. Some of these requirements are for CB banks to report the TNC counts, CD34+ counts, viability, and potency of the CBU. Parameters correlated with measurement of quality of graft and assays used to evaluate the quality/potency of CBUs are stated below.

1.2.1. TNC count

Primarily TNC is the most standard factor in addition to the collected volume of the CBU[19]. TNC count is one of several parameters used to track the graft engraftment potential. Research studies demonstrate that the success rate of engraftment and patient's survival after CB transplantation is greatly correlated on either the number of $CD34^+$ cells per kilogram of the body weight of the recipient or the number of infused TNCs[42,72,51, 67]. Usually, ~ 2-3 $x10^8$ TNC can be harvested from one CBU, while the recommended minimum total number of TNC for HSCT to an adult is 2.5 $x10^7$/ kg[67, 14]. Cryopreservation techniques and processing can also affect the TNC content of CBU and these procedures vary substantially between CB banks. Numerous studies have reported a robust connection between the engraftment activity of CBU and TNC counts[48]. Presently, TNC count is one of the most regulated and reproducible dimensions of the CBU cell dose accessible at the time of selection of graft to describe the potency of CB.

1.2.2. CD34+ cell count

$CD34^+$ cell doses have been correlated with the rate of engraftment after CBU transplantation[43]. Quality of banking is not possible to judge by processing recovery rate

of $CD34^+$ cells primarily, since enumeration of $CD34^+$ cells show large variation between banks[73]. The content of post-processing $CD34^+$ cells should be determined, as it is a good reference for engraftment and potency of CBU. The post-thaw viability and concentration of $CD34^+$ cells can reveal the unexpected cell loss that could occur during cryopreservation[74]. Guttridge, *et al.*[75] also reported that the post- thaw viability of $CD34^+$ cells decreased as a function of storage time due to the increased number of apoptotic $CD34^+$ cells[75]. Therefore, $CD34^+$ cell content after processing the CBU is supportive to measure the graft adequacy. A study conducted by the National Marrow Donor Program and the Center for International Blood and Marrow Transplant Research, jointly with the Histocompatibility Advisory Group, provided evidence-based guidelines for optimal selection of unrelated donors and cord blood units and stated that the minimum CD34+ cell dose for cord blood transplants is 2×10^6 CD34+ cells/kg for single-unit grafts and 1×10^5 CD34+ cells/kg for double-unit grafts[44,53,57,68]. Moreover, it also suggested that a dose ranging from 1.0×10^5 to 1.7×10^5 CD34+ cells/kg at freezing or 1.0×10^5 to 1.2×10^5 CD34+ cells/kg at thawing may be optimal for cord blood transplants[76].

In addition, there are two methodologies used to enumerate $CD34^+$ cells. The technique can differ between banks/processing centers. One technique is the International Society of Hematopoietic and Graft Engineering (ISHAGE) single platform method[66], where a known number of fluorescent beads are added to the sample and the other is dual platform method, in which data from a conventional hematology analyzer and a flow cytometer are combined[77,78]. The single platform method is often favored as it relies on a single measurement. However, Naithani R., *et al.* reported that both methods are effective for $CD34^+$ cell count, and results were comparable between the two methods[79].

1.2.3. CB cell viability

The NetCORD-FACT recommends that the minimum requirement for CBU, is a CD34+ cell viability greater than 70% post-thaw[71]. Research findings demonstrated that the viability of $CD34^+$ cells are overestimated with dye exclusion measurements (example, 7-AAD), which is the primary method used by CB banks around the globe for qualification criteria of CBU. 7-AAD[80,81] permeates the damaged membranes of dead cells only, Annexin V[82,83] stains phosphatidylserine residues which is translocated to the outer membrane in apoptotic cells. Investigations have been done to study the impact of storage using more receptive methods such as Annexin V binding assay that distinguishes apoptotic cells[8485]. They indicated that approximately one in every three $CD45^+CD34^+$ cells can stain positive with Annexin V. Radke, *et al.*[86]and Schwandt, *et al.*[87] showed that evaluation of early apoptotic and necrotic cells by staining with Annexin V and 7-AAD respectively using flow cytometry, is a reasonable technique for calculating the amount of colony forming units (CFUs). Taking this information into consideration, in my research project, I have performed staining with both Annexin V and 7-AAD using flow cytometry, to determine the true impact of CPAs in providing adequate cryoprotection of CB HSCs and HSPCs post thaw.

1.2.4. Potency assays

Another NetCORD-FACT requirement is to demonstrate post-thaw potency through any established potency assay such as colony forming unit (CFU), or other[71]. One of the vital benchmarks in the evaluation of efficacy of processing and cryopreservation is the determination of the regenerative activity of the cryopreserved unit through potency assays. A potency test is a functional assay and represents a quality control that ensures that the HSC product meets the predefined criteria for safety and efficacy before transplantation. Potency is a precise measurement of the biological activity of CB cells,

which allows the production of hematopoietic progenitors[88]. Reduced potency directly correlates to the risk of graft failure[89] and potency can be decreased because of freezing and thawing[90]. The evaluation of CBUs potency predicts the engraftment capacity but it has not been standardized. At present, most CB banks use CFU as their potency assay. Nevertheless, this assay has some disadvantages, such as the 7 to 14 days of incubation before the counting of colonies can be done. This causes delays in CB bank in releasing the CBU for transplantation to the centers[91]. Recently, Interleukin 3 Phosphorylated transducer and activator of transcription-5 (IL-3-pSTAT5) assay has emerged as a competent assay to determine the cell potency in CB banks. In my thesis, I have investigated whether emerging CPAs can provide appropriate cryoprotection on HSPCs by using both the CFU and pSTAT5 potency assays in Aim 1.

1.2.4.1. CFU assay

One of the finest indicators to determine the short-term engraftment potential of grafts is the CFU method[92]. It is considered the gold standard for defining the potency of HSPC grafts[64,90] . The CFU assay is the most widely used as a potency test for HSC banking[93]. CFU assay is performed by plating cells in semisolid methylcellulose with hematopoietic growth factors and appropriate media supplements[42]. Depending on the medium used, different types of colonies can be detected, such as: Colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) which are large, mixed colonies that contain cells from all four myeloid lineages while Colony-forming unit-granulocyte, monocyte (CFU-GM) are smaller, compact colonies that contain cells from the granulocyte and monocyte lineages[94]. Colony-forming unit-granulocyte (CFU-G) colonies are small, tight colonies that contain cells from the granulocyte lineage on the other hand Colony-forming unit-monocyte (CFU-M) are small, loose colonies that contain cells from the monocyte lineage. Both Burst-forming unit-erythroid (BFU-E) and Colony-

forming unit-erythroid (CFU-E) are hemoglobinized clusters that contain cells from the erythroid lineage. The difference is the former ones are larger in size while the latter are smaller[95].

The importance of CFU assay as a potency test for HSC banking is based on several reasons, such as being simple, reliable, and standardized method that can be performed in most laboratories with minimal equipment and expertise[38]. The CFU assay can provide information about the viability, functionality, and diversity of HSPCs in a sample, which can predict the engraftment and recovery outcomes after transplantation. It can detect the effects of various factors that may affect the quality and quantity of HSPCs during banking, such as cryopreservation, thawing, manipulation, contamination, or aging[96]. The number of CFU-GM has been stated to be linked to the transplantation results of HSCs from autologous BM and allogeneic transplantation[96]. Page *et al.* in 2011, confirmed that CFU dose is a strong independent predictor of engraftment after unrelated UCBT and should be used to assess potency when selecting CBUs for transplantation[64,92]. Based on this essential knowledge, the loss of activity of progenitors of CBU can be measured. The inadequacies of CFU assay are associated with the long period of culture (2 weeks), disparity in colony count between individuals, and prerequisite for well-trained trainees/technicians[97].

1.2.4.2. The IL-3- pSTAT5 assay

Given the issues associated with CFU assay, a new potency assay was recently developed by researchers at Hema-Quebec which targets swift usage and reliability for integrating as a routine practice[93,98]. The IL3- pSTAT5 assay is a method that measures the phosphorylation of STAT5, a protein that is involved in the signaling and activation of hematopoietic cells, in response to cytokine such as IL-3 that stimulates the growth and differentiation of HSCs. IL-3 is crucial for stem cell's proper growth, as it promotes multilineage hematopoietic progenitors. IL-3 response is mediated by the JAK/STAT

pathway mediates this IL-3 response which includes STAT5 phosphorylation. It has been shown that STAT5 activation is necessary for augmenting HSC growth and efficacy of engraftment[99].

Besides CB bank setting, this assay has already been proven to be useful in the diagnosis of leukemia and immunomonitoring[91]. The IL3- pSTAT5 assay is a rapid, sensitive, and specific method that can assess the potency and functionality of HSCs. The principle of this assay comprises measurement and detection of STAT5 activation in response to IL-3 as an indicator of HSC potency in CB[90]. A comparison study was conducted and efficacy of IL-3-pSTAT5 assay was evaluated along with CFU and aldehyde dehydrogenase (ALDH) enzyme-based assay. The data obtained revealed IL-3 possessing excellent sensitivity, good identification of variability in CBUs when assessed with CFU assay and was also the most sensitive to detect samples subjected to milder warming events [96]. A recent study conducted by Simard *et al.,* compared the efficacy of PBSCs pSTAT5 assay with CFU and the results indicated that it depicted better specificity[98].

1.3. Xenotransplantation assay

The best technique for detection of functional HSCs is serial cell transplantation in immune deficient mice[100–102]. Transplantation assays are the only available tool for investigating the engraftment capacity of multipotent HSCs having long term self-renewal and differentiating activity. Xenotransplantation models have been extensively employed to study the potential of human cells for initiating and maintenance of the hematopoietic system *in vivo.* Severe combined immunodeficiency (SCID) mouse is a robust model that comes in handy during transplantation. It is considered as the gold standard for xenotransplantation assays to study human HSPC fate *in-vivo*[103]. The non-obese diabetic (NOD)-SCID xenotransplantation model has been used as an early recipient to detect and examine long-term HSC in BM, CB and PBSC transplantation[104]. The NOD-SCID mouse

strain lacks T cells and B cells however, they can develop functional natural killer cells[100,105]. Conversely, loss of IL-2Rγ-chain leads to more defects in innate immunity, so by crossing the IL-2Rγ^{null} gene with conventional SCID and Rag1/2^{null} mice, the NOD-SCID gamma (NSG) (NOD/LtSz-SCIDIl2rg-/-) mouse was developed[100–102,105,106]. In my thesis, I have investigated whether an emerging CPA can provide appropriate cryoprotection on HSPCs function of stemness by using NSG mice in xenotransplantation assay.

1.4. Cryopreservation

1.4.1. Cryopreservation and its characteristics

Cryopreservation is a method that requires the usage of extreme temperatures to preserve structurally intact tissues and cells for prolonged periods of time. It is impossible for any biochemical process to occur at temperatures close to - 196 °C in a liquid nitrogen tank, which in turn permits storage for extended time duration[107]. Cryopreservation is widely used in research, medicine, HSCT, regenerative medicine and upcoming cellular therapy such as chimeric antigen receptor T cells. However, cryopreservation also poses some challenges and risks to the biological material, such as ice formation, cryoprotectant toxicity and cellular damage[108].

Freezing is lethal to most cells due to the formation of ice crystals, that happens at both intra- and extracellular levels, and additionally results in modifications to the chemical setting of cells which results in injuries linked to cellular mechanical constraints[109]. The cryosurvival and cryobiological response throughout the freezing and thawing cycle differ between types of cells. Various kinds of cellular injuries can occur during freezing and thawing[110]. Cell damages leading to cellular death can happen when the cooling rates that are either too high or too low which lead to deterioration of CB quality[111]. It is vital to develop an efficient cell cryopreservation approach, so to get a superior recovery of

functionally viable cells[112]. Selection of a CPA is a crucial step of the cryopreservation process. The right use of CPAs, cooling, and warming rates, along with their concentrations are significant factors determining the efficacy of cryopreservation.

1.4.2. Mechanism of cryoinjury

During any of the cryopreservation process stages, cellular injury can influence cell functionality and/or viability. The length of time from adding the CPA and the sample cooling is significant and is reduced because of the CPA's toxicity[113]. After thawing and preceding to transplantation or transfusion, the CPA may be eliminated to lessen the side-effects upon the product administration. The method of CPA removal frequently requires a sequence of washing and steps of centrifugation[109]. Yang, *et al.,* has validated that elimination of DMSO through numerous washing steps lessen the recovery of CD34+ cells[74] .

The Mazur two-factor hypothesis is a theory that explains how freezing can damage cells during cryopreservation. Mazur's two factor hypothesis presents a suitable description for cellular cryoinjuries[107]. The two factors that can harm cells during freezing are: high salt concentration and intracellular ice formation[114]. The Mazur two-factor hypothesis suggests that different cells have different optimal cooling rates that balance these two factors and minimize the damage caused by freezing. For example, hamster tissue-culture cells have an optimal cooling rate of about -1°C per minute, while yeast cells have an optimal cooling rate of about -100°C per minute[113]. The hypothesis was first proposed by Peter Mazur in 1970 based on his experiments with yeast cells, and later confirmed by Mazur, Stanley Leibo, and Ernest Chu in 1972 using hamster cells. The hypothesis has helped to improve the cryopreservation methods for various types of cells[115]. This hypothesis is elaborately described in the section below.

1.4.2.1. Cryo-injury associated with the freezing

Supercooling effect of cells and extracellular solution occurs at -5°C, but it remains in unfrozen state. Between -5°C and -15°C, nucleation of ice happens in the extracellular medium, but the contents in the cell continue to be unfrozen and in super cooled state. Because of formation of extracellular ice, solutes are omitted from the ice which can lead to rise in solute concentration in the extracellular space. At this point, water gets out from the cell osmotically and freezes outwardly as the system tries to achieve the state of equilibrium[107]. Consequently, cryoinjury relies on the freezing rate employed. If cells are cooled too quickly, the intracellular water of the cell will not egress efficiently to sustain the equilibrium. Also, as there will be swift drop of temperature the intracellular water will freeze and forms intracellular ice. The latter causes damage to cell membrane[113]. On the contrary, if cooling rate is extremely slow, the loss of water will lead to excessive dehydration and intracellular freezing will not occur. This acute loss of volume not only disturbs the membrane but then also exposes the cell to extremely great solute concentrations triggering cell injury. This phenomenon is called solution effect[116]. Consequently, proper optimal cooling rate is essential to avoid intracellular ice formation and to prevent undue cell shrinkage and overcome the solution effect (Figure 4). For each type of cell there is an optimal cooling rate, relying on the cells' membrane permeability to solutes and water. In 1984, Mazur's lab experimentally confirmed this fact[115]. Cooling practices (example- 1–5 °C/min) seem to be in accord amongst most laboratories that involve cryopreservation of CB cells as it permits the cells to steadily dehydrate in reaction to a developing ice phase and rising extracellular solute concentration[117].

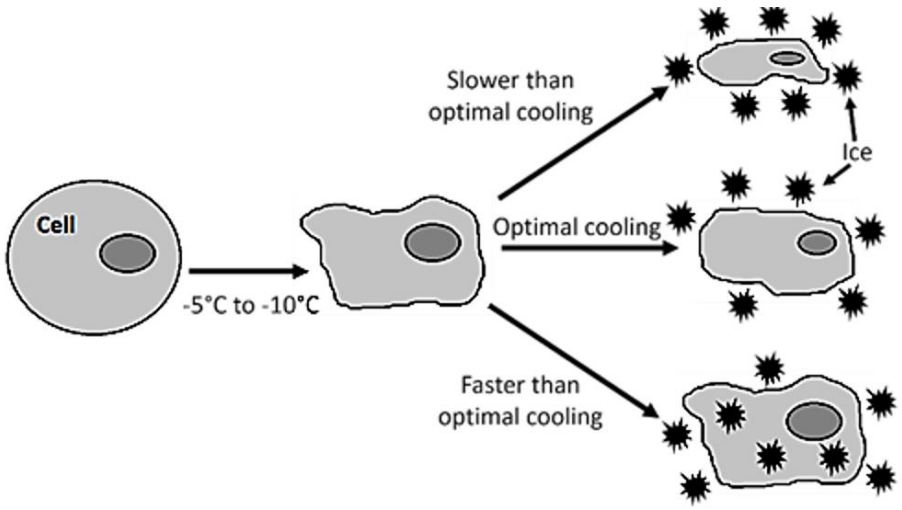

Figure 4: Schematic drawing of physical events in cells during freezing.

Figure adapted from Gao, D., *et al.* 2000[107]

1.4.2.2. Cryo-injury associated with thawing and warming

During the steps of thawing and warming, a cell can encounter different types of cryoinjuries. Thawing done whether at a slow or fast rate can affect the cell negatively leading to mechanical damage and cryo-injury that may include ice recrystallization. At a slow thawing rate, intra and/or extracellular ice crystals will recrystallize into bigger ice crystals which are accountable for the cell membranes rupturing and cause necrosis. Hence, it is imperative to execute speedy thawing of the cells so that ice recrystallization formation is curtailed. Also, intracellular ice recrystallization for the duration of thawing can induce cellular mechanical damage[115]. Nevertheless, it is not possible to eradicate ice recrystallization completely and this kind of cryoinjury is correlated with diminished post-thaw viabilities in various types of cells .CB cell thawing at the speed of around 100 °C/min employing a 37 °C water bath leads to greatest post-thaw viability and recovery of cells[118].

1.4.3. Cryopreservation of CBU

In some situations, fresh stem cells can be used for allogeneic transplants if the cells are transplanted within 72 hours post-harvest. However, virtually all autologous and a

significant proportion of allogeneic HSC transplantations require cryopreservation of HSC grafts[111]. All CBU are frozen until their use in allogeneic transplant settings. Loss of cell viability after cryopreservation can reduce the potency of HSCT. Most importantly, HSCT success correlates with the cell dose and potency[85,90] of the graft. The role of HSCT in the treatment of hematologic and non-hematologic malignancies is rapidly expanding. CBU is a great source of HSCs, but the viability and functional integrity of CB HSPCs should be maintained by proper cryopreservation technique to favor successful transplantation.

CB harvested at the time of birth is stored by cryopreservation in public CB banks for an indeterminate time span. However, graft failure or engraftment delays are major life-threatening issues associated with CB transplants. Slower engraftment is partially due to the significant reduction of neutrophil and platelet engrafting HSPC in CBU vs. adult stem cell grafts[119]. These issues highlight the importance of optimizing cryopreservation of CBU to maximize cell recovery for the successful use of CB[120]. Taken together, the major issues requiring improvement for CB HSCs cryopreservation are, i) improve current cryopreservation protocols, ii) maximize the post-thaw viability and recovery of CB cells and, iii) maximize the post-thaw recovery of progenitor and HSC potencies to promote prompt engraftment. As discussed in more details below, new emerging CPAs represent one strategy that can be explored to improve the post-thaw recovery of CB cells while maintaining significantly greater HSC potency[121]. Therefore, in one of my 1st Aim in my research project, I have characterized the impact of these new cryosolutions for the cryopreservation of CB HSPC.

1.4.4. Types of CPAs and their mechanism of action

CPAs can be categorized based on their chemical structure such as alcohols (glycerol), sugars (trehalose), polymers (polyethylene glycol), sulfoxides (DMSO) and amines (proline). Likewise, CPAs can also be classified based on their localization where

they protect cells from cryoinjury, as intracellular (example- DMSO) or extracellular (example- dextran) CPAs. As previously indicated, CPAs can protect cells through various mechanisms such as by diluting intracellular solutes, preventing large ice crystal formation, preserving cell volumes, and stabilizing cell membranes[122]. The main purpose to use CPAs in combination is to pool their complementary protective properties to further limit cryoinjuries to improve recovery of viable and functional cells post-thaw. Direct inhibition of ice crystal formation and application of antioxidants and other compounds have been investigated to reduce cell death from necrosis and apoptosis during freezing and thawing cycle[30,123].

In natural conditions, many organisms can exist in a dehydrated form for long periods of time through a phenomenon called anhydrobiosis. Small disaccharides like trehalose and sucrose are natural compounds produced by living species to protect from extreme dehydration and cold. Trehalose is a disaccharide of glucose that does not permeate mammalian cell membrane and is considered an osmolyte[124]like proline that can stabilize proteins and cell structures during freezing. Sugars protect cells by stabilizing membranes and proteins by direct interactions through the water replacement hypothesis[118,125] Osmolytes are small solutes usually polar, highly soluble and of low molecular weight. Trehalose has been tested as a CPA with HSC grafts; complementation of DMSO with trehalose has been reported to improve the post-thaw recovery of megakaryocyte progenitors and CFUs, results confirmed in an independent study[126,127]. These findings were extended by others who showed that supplementation with trehalose, pentastarch or sucrose also allowed for DMSO concentration to be reduced to 2.5% to 5.0% [128–130].

Cell cryopreservation demands an optimum cooling rate and the use of CPA, that can lower cell shrinkage during dehydration and impedes intracellular ice formation[125]. Conventional CPAs have lower molecular weight organic compounds that successfully

infiltrate into the cells and inhibit intracellular ice formation, which consist of DMSO, glycerol, propylene glycol and ethylene glycol[128,129]. The cell membrane permeability to water and CPAs is an essential factor defining cell recovery after cryopreservation. CPA used for preserving cells are typically divided into two groups: intracellular/permeating CPAs and non-permeating/extracellular CPAs[123].

Intracellular CPAs like glycerol and DMSO can cross the cell membrane and safeguard cells from cryoinjury correlated with slow cooling rates by lowering "solution effects" and preserving volume of the cell. For the period of slow cooling, cells go through lethally elevated solute concentrations. Penetrating CPAs efficiently dilute these solutes and thus lessen the temperature at which the vital salt concentration is achieved. Simultaneously, penetrating CPAs avoid unnecessary shrinkage of cell by substituting water within the cell[111]. Extracellular CPAs cannot penetrate the cell membrane and work by steadying cell membranes. This kind of CPA mostly forms an armor around the cell, therefore reducing the impact of dehydration because of the freezing procedure. Non-permeating CPAs are comprised of polyvinyl pyrrolidone, polyethylene glycol, sucrose, trehalose, and dextran[109]. They are normally used when rapid cooling rates are employed as they enhance the osmolality of the extracellular solution along with accelerated dehydration[107].

Wu L.K. *et al.,* stated that supplementing CPAs with mono and disaccharides are useful as they have the capability to prevent intracellular ice recrystallization to enhance post-thaw viability of cryopreserved CD34+ cells[131]. Altering pattern formation of ice can affect the freezing response. Consequently, molecules that modify the freezing of water could play a crucial role during cryopreservation[132]. For instance, ice recrystallization inhibitors (IRIs) have been proven to be effective CPAs for cryopreserving HSCs[121].

1.4.5. DMSO as the main CPA for HSC grafts

DMSO has been the foremost choice of CPA for the cryopreservation of HSCs grafts for both adult and neonatal sources. It is readily obtainable in clinical grade preparations and is recognized to be much more penetrable in most cells in comparison to other CPAs such as glycerol. Clinical protocols for cryopreserving CB HSCs typically use 10% DMSO supplemented or not with external CPA such as dextran. For PBSC, both 5% and 10% DMSO are used[98]. Hunt, *et al.* and Rubenstein, et al. stated that utilizing 10% DMSO can sustain high recovery of CFU and very good cell membrane integrity[116,133,134]. In line with this, Meyer *et al.*, also described ideal outcomes with 10% DMSO as revealed by high viability (89%), high recoveries for CD34+ cells (89%) and CFU (88%)[134]. Moreover, DMSO has been adopted as principal CPA for CBU in both clinical practice (CB banking industry) and in most research laboratories. Consequently, in my research thesis, I have used DMSO as a benchmark control to study and compare the efficacy of new CPAs for the cryopreservation of HSPCs (Chapter 2). DMSO was similarly used as gold-standard control in my study of the impact that CPAs may have on key biochemical pathways that support normal cell homeostasis (Chapter 3) and cell response to stress (Chapter 4).

1.4.6. Toxicity of DMSO on cells and humans

Though 10% DMSO solution is very efficient and retains elevated levels of viability for most hematopoietic cells, there are several substantial disadvantages linked with the usage of DMSO[108,135,136]. Several mild side-effects have been related with DMSO transfusion into patients involving nausea, headache, vomiting, hypertension[137]. Also, in some very rare cases, DMSO toxicity can cause fatal arrhythmias, respiratory arrest, seizures, and hemoglobinuria[138]. Notably, a retrospective analysis of intravenous DMSO toxicity in transplant patients revealed that the overall incidence of DMSO toxicity was

approximately one in 70 transplants, and the mean incidence per center was 2.1%[134]. Additionally, gastrointestinal and skin reactions are the commonest reported adverse reactions to DMSO[139].

It is known that the extent of toxicity is associated with the quantity of DMSO transfused in the patient. Thus, efforts have been made to decrease the amount of DMSO preceding to its transfusion[127]. One approach is to reduce the concentration of DMSO for cryopreservation. In line with this, a study reported that the combination of 5% DMSO with pentastarch provided higher post-thaw viability than control CB cells cryopreserved with 10% DMSO[140]. Another approach is to remove DMSO from the grafts through washing steps. However, this method is costly, lengthy, and often leads to cell loss[141]. A study found that washing with HES solution was more effective than dilution with saline and improved the recovery of CB TNCs, CD34+ cells, and CFUs[87], though others saw diminished CD34+ cells recovery and viability[73]. Alternatively, graft can be diluted to simplify the processing and avoid cell losses[138]. In my work, I adopted the same thaw protocol developed in our lab for the CB bank, which involves two dilution steps to provide time to adjust to the change in osmolarity and avoid excessive cell swelling that occur upon rapid dilution[108].

Several studies have also reported on the cytotoxicity of DMSO in hematopoietic cells. Under hypothermic conditions, DMSO has a low cytotoxic effect even after 24 hours of incubation, but the viability of the cells decreased significantly at room temperature and 37°C[142 143]. A study on the effect of DMSO concentration on the viability and function of cryopreserved PBMCs found that DMSO concentrations above 5% significantly reduced the viability, proliferation, and cytokine production of PBMCs[94,144]. Also, DMSO concentrations above 10% were shown to impair the expression of surface markers and the cytotoxic activity of NK cells[145]. DMSO has been demonstrated to have chemical toxic

influences on cells causing damage of proliferative capability of hematopoietic cells including HSPCs when present at concentration greater than 0.25% (range 0.1-0.5%) for extended period[138]. In summary, the temperature, and the duration of DMSO exposure should be carefully controlled to minimize cell damage[135,136]. As such, most DMSO-based cryopreservation protocol are done with chilled cells and cold DMSO solution.

DMSO has been shown to elicit epigenetic changes resulting in changes in human cells[87,134,146]. A recent study reported the effects of DMSO exposure on 3D cardiac and hepatic microtissues. The results showed that DMSO induced global DNA hypomethylation, alters histone acetylation and methylation patterns, deregulated microRNAs, and affected cellular processes such as cell cycle, apoptosis, and metabolism[146] According to a latest study that investigated the influence of DMSO on liver-specific gene expression in HepaRG cells, DMSO modulated the expression of genes involved in drug metabolism, bile acid synthesis, lipid metabolism, and inflammation. Moreover, DMSO also affected the DNA methylation and histone acetylation status of some hepatic-specific genes[87].

Even though DMSO is a safe and effective CPA, there remains room for improvement at least for the cryopreservation of HSPCs. In fact, several studies have shown that up to 30% of CD34+ HSPCs are apoptotic post-thaw,[84,108] which has been correlated with a decreased engraftment activity[108,126]. The detection of apoptotic cells which require annexinV staining is not carried out in clinical settings and thus this fact is certainly underreported. Nearly 20% of patients obtaining a CB transplant will fail to engraft, majorly due to the insufficient potency of the cryopreserved unit[90]. The above issues support that further optimization is needed for cryopreservation of HSPCs and determine whether new DMSO-free CPAs could be useful to improve the efficacy of HSPCs post thaw.

1.4.7. Development of DMSO-free CPA solutions

The identification of new CPAs has paved the way for the development of new commercial solutions in the likes of CryoStor (BioLife Solutions), which can be used with lower DMSO concentration with HSC grafts[147]. Indeed, it was reported that the addition of CryoStor (i.e., final concentration ≤5% DMSO) resulted in significantly better post-thaw recovery of CB CD34 + cells[148]. In 2016, successful cryopreservation of PBSC grafts without DMSO was reported with pentaisomaltose (PIM), a 1-kDa subfraction of Dextran 1. The recovery of PBSC CD34 + cells and CFUs was equivalent between grafts frozen with 10% DMSO or 16% PIM[149]. More importantly, in a follow up study with xenotransplant experiments, they showed that human engraftment was retained in PIM-protected grafts. To my knowledge, this was the first demonstration that HSC graft could be cryopreserved without DMSO and maintain high engraftment activity.

Since then, several new DMSO-free solutions have become commercially available, these include Cryoscarless (CSL, Diagnocine), CryoSoFree (MilliporeSigma), CryoNovo (Akron Biotech), StemcellKeep (Diagnocine) and CryoProtectPure (CPP, Ad Infinitum Cell Preservation Technologies - Spectacular Diagnostics). CSL is a xenogeneic- and serum-free medium formulated for the long-term storage of cells at -80°C or in liquid nitrogen. Successful cryopreservation of ex vivo-expanded CB T cells[150] and nematode microfilaria[151] was recently reported with CSL. On the other hand, CN is manufactured entirely from naturally occurring CPAs. It has been shown to be superior to DMSO-containing cryopreservation media when used with mesenchymal stromal cells (atlantisbioscience.com). Finally, CPP is a new DMSO and serum-free balanced salt-based formulation that contains glycol derivatives with known cryoprotective abilities and molecular-grade, non-toxic protein components of xenogeneic origin with low

immunogenic implications. Moreover, StemCellKeep uses vitrification which is not yet feasible for CBU as discussed above.

Given the infusional toxicity associated with DMSO and its potential impact on cellular toxicities in HSCs, the suitability of new emerging DMSO-free CPAs for the cryopreservation of HSPCs was investigated with two key aims; 1) to improve HSPC viability and potency following cryopreservation; and 2) to determine whether DMSO-free solutions represent a suitable alternative to DMSO for the cryopreservation of HSPC grafts. As such, in my 1st aim of my thesis I have investigated the cryoprotective properties of several new CPAs to identify to characterize their cryoprotective properties on HSPCs, benchmarking them against a clinical-grade DMSO/dextran-40 cryopreservation solution.

1.4.8. Role of bioenergetic pathways in HSC cryopreservation

1.4.8.1. Bioenergetic pathways

Metabolic mechanisms and pathways are a complicated set of regulated biochemical reactions that transform energy substrates into adenosine triphosphate (ATP) and other metabolic building blocks[152]. Bioenergetic pathways are the processes that cells use to produce and regulate energy in the form of ATP. These pathways include glycolysis, oxidative phosphorylation, fatty acid oxidation and ketogenesis[99]. These pathways are essential for maintaining cellular homeostasis, survival, and function. Different types of cells and tissues may prefer different bioenergetic pathways depending on their energy needs, oxygen availability, metabolic substrates, and environmental conditions[153].

Glycolysis is the process of breaking down glucose into pyruvate, which produces energy in the form of ATP and nicotinamide adenine dinucleotide (NADH). Glycolysis can occur in both aerobic and anaerobic conditions, but in the latter case, pyruvate is converted into lactate to regenerate NAD+ for glycolysis to continue[154]. Glycolysis is regulated by several enzymes, such as hexokinase, phosphofructokinase, and pyruvate kinase, which are

influenced by the levels of ATP, NADH, glucose and other metabolites[155]. It is a fast but inefficient way of producing energy, and it is mainly used by cells that have high energy demands or low oxygen supply, such as muscle cells, brain cells and red blood cells[156].

Oxidative phosphorylation is the process of producing energy in the mitochondria by transferring electrons from NADH to oxygen through a series of protein complexes called the electron transport chain[78]. This creates a proton gradient across the inner mitochondrial membrane, which drives the synthesis of ATP by the enzyme ATP synthase. It is regulated by the availability of oxygen, NADH and adenosine diphosphate (ADP), as well as by the activity of uncoupling proteins, which can dissipate the proton gradient as heat[157]. This process may be slow but is efficient for producing energy, and it is mainly used by cells that have low energy demands or high oxygen supply, such as liver cells, kidney cells and heart cells[158]. Dysregulation of these pathways plays a significant role in the development and progression of ailments like obesity, chronic metabolic diseases such as type 2 diabetes and autoimmune disorders like rheumatoid arthritis[157].

Generation of ATP is majorly through oxidative phosphorylation in the mitochondria and glycolysis located inside cytosol. Glycolytic and mitochondrial ATP generation rates are driven by metabolic reactions[154]. Oxygen consumption in the oxidative phosphorylation pathway is necessary for the oxidation of decreasing equivalents produced from pyruvate and other energy substrates, leading the mitochondrial ATP production[156]. Hence, precise measurement of oxygen fluxes and assessment of proton fluxes[155,159] can indicate the source and rate of cellular ATP production. In assays pertaining to extracellular flux, measurement of extracellular acidification rates (ECAR) and cellular oxygen consumption rates (OCR) are simultaneously performed in real-time using fluorescent sensors in an apparatus called, Seahorse extracellular flux (XF) analyzer, is a powerful tool in analysis of bioenergetics of cells[157]. As described in more details below, I have used the

Seahorse technology to study the impact of CPAs on mitochondrial and glycolytic pathways in HSPCs.

1.4.8.2. Bioenergetic mechanisms in HSCs

The bioenergetic pathways of HSCs are important for regulating their fate decisions, such as quiescence, self-renewal, and differentiation[160]. Mitochondria are involved in various metabolic and signaling processes that affect the function and fate of HSCs[161]. Studies have indicated that mitochondria in HSC can impact homeostasis and provide a balance between quiescence, self-renewal, and differentiation of HSCs, depending on the physiological and environmental cues[162–164].

Quiescent HSCs have low energy requirements and are thought to rely mainly on glycolysis but not mitochondria as their source of energy sustained by hypoxic niches[165]. They have a relatively high number of mitochondria that are mostly inactive and produce low levels of reactive oxygen species (ROS), which are harmful molecules that can cause oxidative stress and DNA damage[166]. HSC resides in hypoxic niches in the BM, where the oxygen levels are low and mitochondrial activity is suppressed. This helps to maintain the low-energy and low-oxidative state of HSCs, which preserves their long-term self-renewal and multipotency[164]. Glycolysis regulates the expression and activity of some key factors that control HSC fate, such as hypoxia-inducible factor 1α (HIF-1α), mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1)[165].

In summary, glycolysis plays a crucial role in maintaining HSC homeostasis by providing a metabolic balance between quiescence and activation, self-renewal, and differentiation, and multipotency and commitment[163]. When HSCs enter cell cycle and/or undergo differentiation, they switch from glycolysis to oxidative phosphorylation[167]. This switch is associated with an increase in ROS, mitochondrial biogenesis, oxygen

consumption and metabolic regulators[156]. Oxidative phosphorylation provides more energy for HSCs to support their proliferation and differentiation, but also exposes them to higher oxidative stress and DNA damage that may compromise their function or induce senescence or apoptosis[168]. Therefore, mitochondria play a crucial role in maintaining HSC homeostasis by providing a metabolic balance between quiescence and activation, self-renewal, and differentiation, and multipotency and commitment[169].

1.4.8.3. Impact of cryopreservation on bioenergetic pathways

Unknown to many, is the fact that cryopreservation affects bioenergetic pathways in cells[79]. Cryopreservation does alter the expression and activity of enzymes and genes involved in these pathways, as well as the levels of metabolites and cofactors[145]. This may result in changes in the energy production and consumption of the cells, as well as their oxidative stress and mitochondrial function[109]. A research study done using primary T cells showed for the first time that during cryopreservation modified their metabolism in a time-dependent trend[157]. Taking these findings into consideration, a better understanding concerning the relationship between cryopreservation and CPAs is necessary[119], [89] .

A study by Abazari *et al*., investigated the effect of cryopreservation on bone marrow-derived mesenchymal stem cells (BM-MSCs), and observed that cryopreservation altered the expression of genes related to bioenergetic pathways, such as glycolysis, oxidative phosphorylation, and fatty acid oxidation[170]. They also found that cryopreserved BM-MSCs had lower levels of ATP and higher levels of ROS than fresh BM-MSCs. This indicates that cryopreservation may impair the bioenergetic status and oxidative balance of BM-MSCs, which may affect their therapeutic efficacy[170,160]. A study by El-Sayed *et al.,* investigated the effect of cryopreservation on BM-MSCs. The authors compared the phenotypic and functional traits of fresh and frozen-thawed BM-MSCs, and found that cryopreservation did not affect their viability, morphology, immunophenotype or

differentiation potential. However, cryopreservation altered the expression of genes related to bioenergetic pathways, such as glycolysis, oxidative phosphorylation, and fatty acid oxidation. They also found that cryopreserved BM-MSCs had lower levels of ATP and higher levels of reactive oxygen species (ROS) than fresh BM-MSCs[171].

During cryopreservation, mitochondria is one of many organelles affected by osmotic shock, extreme cold and oxidative stress resulting in apoptotic-like modifications[172]. The oxidative role of mitochondria is a crucial consideration to understand metabolism in healthy and defective cells. Mitochondria uses 90% of the oxygen that is inhaled via Complex IV of the electron transport chain (ETC)[153]. The ETC is very crucial for the transformation of energy of nutrients into a proton gradient used for ATP generation. Furthermore, it is essential for all other characteristics of cellular metabolism relying on mitochondria. Therefore, it is vital to measure oxygen consumption rate, which is the gold standard in measuring the functionality of mitochondria[173]. Interestingly, a recent bioinformatics analysis discovered that pathways like glycolysis or gluconeogenesis, the citrate cycle (TCA cycle), pyruvate metabolism and galactose metabolism play an essential regulatory role in process of cryopreservation[89,174].

In summary, several studies have shown that HSC favor glycolysis over oxidative phosphorylation. Also, other studies have shown that cryopreservation can impact bioenergetic pathways. However, the effect of CPAs on bioenergetic pathways remains unknown. Therefore, in my research project I have studied the impact of different CPAs including the gold standard DMSO on bioenergetic pathways in HSPCs.

1.4.9. Role of Autophagy in cryopreservation

1.4.9.1. Autophagy-mechanism and functions

Autophagy is a process by which cells degrade and recycle their own components, such as proteins, organelles, and cytoplasmic material. Autophagy has many functions in

cells, such as providing energy and nutrients during starvation or stress, removing damaged or dysfunctional organelles and proteins, regulating cell growth and differentiation, modulating immune responses and inflammation, controlling cell death and survival preventing diseases, such as cancer, neurodegeneration, infection, and aging[175,176] Induction of autophagy occurs as a response to both intracellular (e.g.increase of damaged organelles, accumulation of proteins) and extracellular stress situations (e.g. hypoxia, nutrient deficiency, and oxidative stress).

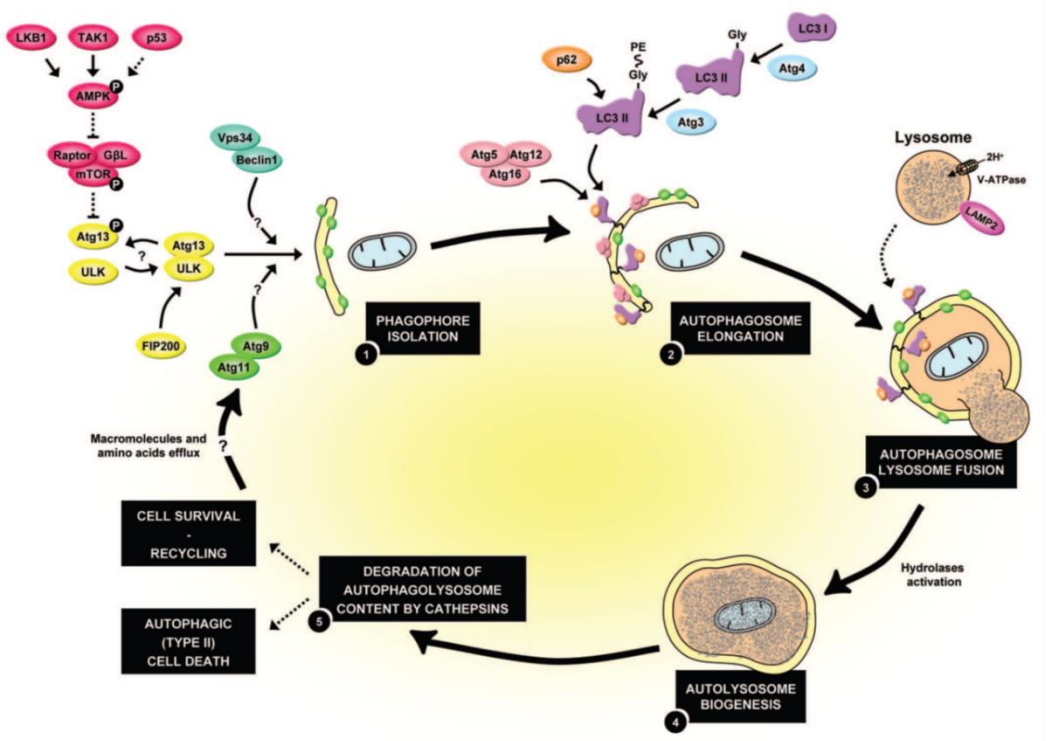

Figure 5: A schematic molecular view of autophagy.

Autophagy can be subdivided in three main steps as depicted in the figure. Figure adapted from Alexandre., *et al*. 2010[177]

Even though autophagy and autophagy-related procedures are dynamic in nature, they can be divided into separate phases: (1) induction, (2) autophagosome formation, (3)

autophagolysome formation (4) delivery and degradation of the autophagic body[178,179] (Figure 5). The first step in autophagy is the formation of a membrane structure called the phagophore, which originates from various sources, such as the endoplasmic reticulum, mitochondria, or plasma membrane. The phagophore is regulated by a complex of proteins called the ULK1 complex, which is activated by nutrient deprivation and inhibited by mTOR signaling. The second step in autophagy is the expansion of the phagophore membrane to engulf the cytoplasmic cargo. This step requires two ubiquitin-like conjugation systems: the ATG12-ATG5-ATG16L1 complex and the LC3 (or ATG8) system. These systems attach lipid molecules to the autophagy proteins, which then recruit them to the phagophore membrane[32].

The third step in autophagy is the closure of the phagophore membrane to form a double-membrane vesicle called the autophagosome. The autophagosome can selectively sequester specific targets, such as damaged organelles, protein aggregates, or pathogens, by using receptors that recognize specific signals on the cargo. For example, p62 (or SQSTM1) is a receptor that binds to ubiquitinated proteins and LC3 on the autophagosome membrane[180]. The fourth step of autophagy is the fusion of the autophagosome with a lysosome, which is an organelle that contains digestive enzymes. The final step of autophagy is the degradation of the cargo inside the autolysosome, which is the product of the fusion between the autophagosome and the lysosome. The lysosomal enzymes break down the cargo into basic molecules, such as amino acids, fatty acids, and nucleotides, which are then released into the cytosol for reuse or disposal[36].

Autophagy is controlled by the autophagy-related genes (Atgs), the products of which orchestrate the formation of double-membrane vesicles that capture and sequester intracellular components[181]. The principal player in signaling pathways of autophagy is mammalian target of rapamycin (mTOR), which negatively controls autophagy. mTOR

creates two distinct multi-protein complexes, which are represented as mTOR complex 1 (mTORC1) and mTORC2[167]. mTOR is very well conserved from yeast to mammals[182]. As an important cell growth and metabolism regulator, mTOR regulates growth related procedures like aging, development, and hypoxia responses[183]. Hence, mTOR inhibitors might be beneficial for the therapeutic strategies of human illnesses like metabolic diseases and neurodegenerative disorders.

Autophagy is known to be activated by biochemical mechanisms in response to stress and it generates energy through an independently regulated catabolic process[177,178]. However, no study has investigated the role that autophagy has in stem and progenitor survival following cryopreservation[184]. Therefore, in this thesis project I have investigated the link between autophagy and cryopreservation of HSCs and HSPCs.

1.4.9.2. Autophagy modulators

Autophagy modulators are substances that can modulate the process of autophagy and those have been shown to have various effects on cell function and in more complex processes such as inflammation and disease to name a few[185].

Autophagy activators stimulate autophagy through various mechanisms, such as inhibition of the mTOR pathway which is a negative regulator of autophagy[34]. Rapamycin, metformin, and resveratrol are autophagy activators that act as mTOR inhibitors[186]. Others act through AMPK pathway activation like berberine, and curcumin[179]. AMPK inhibits mTOR activity[174]. Trehalose, on the other hand is a natural sugar that has been shown to have beneficial effects on the brain and other organs by modulating autophagy. The exact mechanism of its activation role in autophagy is however unclear[187]. Although some research studies have shown that trehalose can reduce the activity of mTOR by lowering the levels of insulin and glucose in the blood[188,189], increase the activity of AMPK by enhancing the production of ROS in the mitochondria[188].

Autophagy inhibitors interfere with the process of autophagy in cells through different mechanisms, like by inhibiting the formation or expansion of the phagophore. 3-methyladenine (3-MA), wortmannin, and LY294002 are some of the commonly known phagophore inhibitors[190]. They may act by inhibiting the conjugation or recruitment of autophagy proteins[191]. Autophagy late-stage inhibitors such as bafilomycin A1 and spautin-1 act through this mechanism.

The presence of autophagy modulators (activators or inhibitors) enables the analysis of autophagy for its function and therapeutic capability in human illnesses[173,181]. Herein, in this research project, I have tested the importance of autophagy in cryopreservation, and see if I could improve potency through autophagy modulation in CB HPSCs.

1.4.9.3. Autophagy and HSC homeostasis

HSCs need a fine balance between quiescence, proliferation, and differentiation to maintain their stemness and hematopoietic homeostasis[180]. Autophagy is involved in the regulation of these HSC fates by modulating various signaling pathways, such as mTOR, AMPK, p53, and NF-kB1[192]. Moreover, autophagy can promote HSC quiescence by inhibiting the mTOR pathway, which is a negative regulator of autophagy and a stimulator of HSC proliferation and protect HSCs from oxidative stress and DNA damage by removing damaged organelles and proteins[193]. Quiescence is essential for HSC survival and function throughout life. Autophagy can also support HSC proliferation by providing energy and nutrients during stress conditions, such as nutrient deprivation or infection[194]. Autophagy can also regulate the cell cycle and apoptosis of HSCs by modulating the p53 pathway, which is a tumor suppressor and a regulator of autophagy[195]. Autophagy can influence HSC differentiation by affecting the expression and function of transcription factors, such as PU.1, C/EBPα, GATA-1, and FOXO3a, which are involved in the lineage

commitment of HSCs[192]. It may modulate the epigenetic status of HSCs by regulating histone acetylation and methylation[196].

An imbalance in the equilibrium between the differentiation and self-renewal properties of HSCs result in different illnesses associated with hematopoiesis. Hematopoietic cells transform into leukemia cells at the stem cell or progenitor cell stage[197]. Warr., *et al.* in 2013, demonstrated that autophagy as a crucial mechanism to protect HSCs from metabolic stress. The results in this study validated that FOXO3A is essential to sustain a gene expression program that balance the flow of HSCs for quick autophagy initiation upon starvation. Particularly, the findings suggests that old HSCs maintain an unbroken FOXO3A-driven pro-autophagy gene system, and that continuing autophagy is vital in alleviating an energy predicament and permit their endurance[198]. Research studies have discovered that levels of CD147, a transmemberane protein, rise in cancer cells, causing tumor progression. Isabella *et al.* discovered that AC-73, which is an inhibitor of CD147, reduces cell proliferation of leukemia due to the inhibition of the extracellular-signal-regulated kinase (ERK)/signal transducer and activator of transcription 3 (STAT3) pathway and autophagy activation[192]. Furthermore, autophagy has been observed to impact the homeostasis of adult stem cells derived from bone marrow regarding their stages of quiescence, differentiation, and self-renewal[199]. Together, all these findings suggest that autophagy has significant contribution in impacting HSC homeostasis[193].

Besides anaerobic glycolysis, autophagy, especially mitophagy (a particular term for autophagy associated with mitochondrial elimination), likewise plays an essential role in self-renewal ability of HSC. Dysfunctional and unwanted mitochondria are removed and salvaged through mitophagy to support the stemness of HSCs[174]. This results in oxidative stress, which leads to the rise of production in ROS thereby, limiting the potential for self-

renewal which in turn leads to the conversion of long-term HSCs to short-term HSCs[200]. A research study conducted within *ATG12* knockout mouse model, indicated an increase in mitochondrial production and faster oxidative phosphorylation which led to enhancement of HSCs entry into the cell cycle[197]. In 2010, Liu *et al.,* reported that enhanced ROS levels and mitochondrial mass in FIP200-deficient fetal HSCs inhibited their regulation[201]. Correspondingly, knockdown of *ATG7* in HSCs directs the buildup of superoxide and mitochondria, resulting in malfunctioning of differentiation into myeloid- and lymphoid-precursor cells[202].

1.4.9.4. Impact of cryopreservation on autophagy

The impact of cryopreservation on autophagy has been studied in various cell types and tissues, such as spermatozoa, oocytes, ovarian tissue, BM-MSCs, and hepatocytes[123,132]. However, some studies suggest that cryopreservation can either activate or inhibit autophagy depending on the cell type, the cryopreservation method, and the cryopreservation duration[77, 175, 176, 203]. For example, cryopreservation by vitrification increased the expression of autophagy-related proteins (such as LC3 and Beclin-1) and the formation of autophagosomes in ovarian tissue and oocytes[202]. Cryopreservation by slow cooling also increased the expression of autophagy-related genes (such as *ATG5* and *ATG7*) and the activity of lysosomal enzymes (such as cathepsin B) in hepatocytes[181]. These studies suggest that autophagy activation can help protect cells from cryoinjury by removing damaged organelles and proteins.

However, other studies have also shown that cryopreservation can inhibit autophagy in cells because of cryoinjury or cryoprotectant toxicity[134]. For example, cryopreservation by slow cooling decreased the expression of autophagy-related proteins (such as LC3 and Beclin-1) and the formation of autophagosomes in spermatozoa1 and bone marrow-derived mesenchymal stem cells[203,204]. Cryopreservation by vitrification also

decreased the expression of autophagy-related genes (such as ATG5 and ATG7) and the activity of lysosomal enzymes (such as cathepsin B) in oocytes[134] . These studies suggest that autophagy inhibition can impair cell function and quality by accumulating damaged organelles and proteins during cryopreservation.

As mentioned before, CPAs are important components in cell cryopreservation. Trehalose, a nonreducing and natural disaccharide, has the capability to protect cells as a CPA. Owing to its role of an autophagy activator, trehalose can impact on cell cryopreservation because of this mechanism[130]. A recent research study revealed impact of autophagy on cryopreservation by using cryopreserved human aortic endothelial cells in various cryopreservation fluids with or without DMSO and trehalose was added[122]. The data indicated that, the expression of the autophagy-related genes *BECN, LC3* was raised by trehalose and reduction of the expression of P62 was observed. Analysis also demonstrated that cell death and apoptosis were decreased through cryopreservation with trehalose. The data implied that there could be a correlation between trehalose-initiated autophagy and the defensive role of trehalose in cryopreservation[205, 152]. Conversely, basal level of autophagy was shown to be vital for flexibility in cryopreserved murine spermatogonial stem cells post thaw[175, 176].

As stated above, studies have revealed that autophagy is vital for the life-long preservation of the HSC[206]. Also, the impact of cryopreservation on autophagy is controversial, complex, and variable depending on various factors. More studies are needed to explore the potential role of autophagy during cryopreservation and whether modulation of autophagy could improve cell survival and function. Toward this, I will examine the impact that cryopreservation and different CPAs have on autophagy in HSPCs, and whether autophagy is required for successful freezing and thawing of CB HSPC cryopreservation.

1.5. Rationale, Hypothesis and Aims

Rationale

CB has emerged as an attractive source of HSCs for allogeneic transplantation for patients without matched donors due to rare or complex HLA complexes[47]. Cryopreservation is the only method available for the preservation of CBU and other HSPC grafts for long periods. Loss of cell viability post-thaw has been shown to reduce potency and lead to poor outcomes in fundamental and clinical studies[5]. Current cryopreservation protocols with DMSO do not protect CB cells and HSPCs completely from cryoinjuries leading to cell loss and reduced potency post-thaw[207]. Moreover, DMSO has been shown to be toxic in most cells including HSPCs[110,111] and elicit epigenetic changes in human cells resulting in property changes[134,137,138,146].

Interestingly, new CPA solutions have now been developed including several devoid of DMSO. It is unclear at this time and important to investigate if they can be applied to HSPCs as they may offer improved cryoprotection. In addition, it is equally crucial to understand why some CPA may work well with HSPC and others not. Such strategy was undertaken to identify not only new CPA solutions of interest to improve HSC graft cryopreservation, but also to identify pathways affected by CPAs that could perhaps in the future be modulated to enhance post-thaw HSPC viability and potency.

Therefore, in aim 1, I have investigated the effect of 4 CPA solutions and benchmarked them against DMSO used as standard control. The protective properties of those CPAs in HSPCs were characterized using a series of *in vitro* and *in vivo* phenotypic and functional assays. In aim 2, I have studied the impact of these CPAs on key biochemical pathways important for cell homeostasis by assessing their impact on OXPHOS and glycolysis, and on HSPC' cell membrane leakiness and lipid peroxidation. Finally, in aim 3, I have investigated the impact of cryopreservation and CPAs on autophagy, an important

mechanism used by cells to sustain viability and potency in response to stress. Together, my 3 aims provide a full circle investigation of the impacts of CPAs and cryopreservation on biological processes sustaining CB cell and HSPC' survival and potency. Together, my findings enhance our knowledge of new CPAs for HSPCs and pathways that could be modulated in the future to perhaps improve the cryopreservation of HSC grafts.

Hypothesis

My overarching hypothesis is that different CPAs will have diverse impact on the key biochemical processes essential to HSPCs homeostasis (such as bioenergetic pathways and autophagy) which will influence their capacity to protect HSPCs from cryoinjuries and sustain elevated post-thaw cell viability and potency.

Aims & Objectives

Aim 1- Evaluate the cryoprotective properties of different CPA solutions in CB cells.

Rationale: Recent advances in the field of CPAs and cryobiology have led to the development of new cryosolutions including some devoid of DMSO. However, their ability to preserve HSPCs has for most not been investigated at the current time. While the exact make up of those solutions is not fully disclosed, it is still important to assess their usefulness in protecting stem cell grafts from cryoinjuries since they could be an attractive alternative in the future to DMSO. Such CPA should be safe for use in humans, support post-thaw viability equal to or superior to DMSO, and efficiently protect HSPC from cryoinjuries to sustain long-term engraftment. Hence, I investigated the cryoprotective properties of several new CPAs to characterize their cryoprotective properties on HSPCs.

Objective 1.1: Characterize the cryoprotective properties of new CPAs on CB cells and HSPCs.

***Objective 1.2**:* Investigate the impact of selected CPAs on the proliferation and differentiation abilities of cryopreserved HSPCs.

Objective 1.3: Determine the effect of selected CPAs on engraftment activity of HSC graft by functional validation through xenotransplantation assay.

Aim 2- Investigate how different CPAs impact mitochondrial respiration, glycolysis, and cell membrane integrity of HSPCs.

Rationale: In aim 1, I identified CPAs like CPP and CSL that efficiently cryoprotected HSPCs while others like CN failed to. In aim 2, I investigated how these CPAs impact key biochemical mechanisms known to be important for cell survival. Recent evidence suggests that biological processes such as glycolysis and mitochondrial respiration may be affected during cryopreservation[155]. Given the range of impact on cell viability and potency of HSCs using novel CPAs in Aim 1, this provides an opportunity to distinguish the differential impact of key bioenergetics pathways and membrane stability. Mechanistic insight regarding CPA effectiveness may identify key targets for future development of optimal CPAs. Therefore, in this aim, I sought to investigate the impact of CPAs on mitochondrial respiratory pathway and glycolysis cycle in HSPCs in context of cryobiology. Furthermore, I studied the short-term effect of these CPAs on hematopoietic cell membrane before freezing.

Objective 2.1: Study the impact of CPAs on mitochondrial respiration and glycolysis of HSPCs.

Objective 2.2: Evaluate the influence of CPAs on hematopoietic cell membrane integrity and lipid peroxidation.

Aim 3- Elucidate the role of autophagy during the cryopreservation of HSPCs.

Rationale: Several studies have now revealed that autophagy is important for the regulation and function of HSPCs[32,77,93]. Besides, autophagy is known to be of vital importance to sustain cell viability under non-homeostatic conditions or stress[180,185]. However, the role of autophagy in HSPCs following cryopreservation remains unknown and, the impact that CPAs may have HSC autophagy remains unclear. Thus, to complement my findings on the characterization of new CPAs in HSPCs (Chapter 2) and on the impact that these CPAs have on bioenergetics and cell membrane of HSPCs (Chapter 3), I set out to characterize the impact of CPAs and cryopreservation on autophagy in HSPC.

Objective 3.1: Investigate if autophagy is activated within HSPCs during cryopreservation.

Objective 3.2: Define the importance of autophagy during cryopreservation of HSPCs.

Objective 3.3: Determine if CPAs can influence autophagy in HSPCs.

CHAPTER 2

CPP IS A NOVEL CPA THAT HAS SIMILAR CRYOPROTECTIVE ABILITY TO THAT OF DMSO[1]

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FULL-LENGTH ARTICLE
Basic Research

Dimethyl sulfoxide-free cryopreservation solutions for hematopoietic stem cell grafts

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[1] Based on an article "Dimethyl sulfoxide-free cryopreservation solutions for hematopoietic stem cell grafts" published in Cytotherapy in 2022. First author Richa Kaushal participated in all experimental designs, CB processing, *in vitro* assays, mice transplantation assays, data analyses and wrote the manuscript. Suria Jahan assisted in viability post thaw analysis and *in vivo* mice transplantation assay. Chelsea McGregor performed confirmatory *in vitro* viability and potency post thaw analysis using ISHAGE technique. Nicolas Pineault conceived the study, supervised the project, and edited the manuscript.

2.1. Summary

Background and aims: The use of effective methods for the cryopreservation of HSCs is vital to retain the maximum engraftment activity of CBUs. Current protocols entail the use

of DMSO as an intracellular CPA and dextran and plasma proteins as extracellular CPAs, but DMSO is known to be cytotoxic, and its infusion in patients is associated with mild to moderate side effects. Although new CPAs have been developed, their capacity to protect HSCs remains poorly investigated.

Methods: Herein I compared the capacity of four DMSO-free freezing media to cryopreserve CB HSCs: CPP, CSL, CN and PIM. Clinical-grade DMSO/dextran solution was used as control. The CPAs were screened to measure their ability to recover TNC number, viability and HSPC potency post thaw. The viability was measured using FACS and HSPC potency via CFU assay. The DMSO and CPP frozen CD34-enriched cells were then tested in a liquid-based HSC expansion assay. Xenotransplantation assays were performed to compare the engraftment activities of CB TNCs (i.e., CB buffy coat cells) cryopreserved with CPP or control DMSO. Engraftment analyses were first done by tracking human cells in the periphery at week 4, week 10 and week 22. Cell populations were tracked and analyzed using cell surface markers.

Results: Of the four cryopreservation solutions tested, the best post thaw cell viability, recovery of viable CD45+ and CD34+ cells and potency were achieved with CPP, which was equal or superior to that seen with the control DMSO. CSL provided the second-best post thaw results followed by PIM, whereas CN was associated with modest viability and potency. Further work with CPP revealed that CB CD34-enriched HSCs and progenitors cryopreserved with CPP maintained high viability and growth expansion activity. In line with this, a pilot transplantation assay confirmed that CPP-protected CB grafts supported normal short- and long-term engraftment kinetics.

Conclusions: The results suggest that new, valuable alternatives to DMSO are now available for the cryopreservation of HSCs and grafts, including CBUs.

2.2. Introduction

Cryopreservation is one of the best means of preserving (HSCs) grafts for transplantation. Nevertheless, even with standard cryopreservation protocols and cell-permeating CPAs such as DMSO, substantial cellular damage still happens, which reduces post thaw cell viability and weakens the functional activity of the grafts. Cell damage during freezing and thawing is a result of osmotic pressure, dehydration, and uncontrolled growth of ice (recrystallization). The potential unfavorable effects of this phenomenon can be avoided by using effective extracellular and intracellular CPAs together with proper cooling rates[115]. Nonetheless, advancements in cryopreservation processes are needed to minimize side effects in patients, maximize graft potencies and pave the way for upcoming new cell-based regenerative therapies.

UCB emerged as an alternative source of HSCs for allogeneic transplantation after the first CB transplantation was successfully performed in 1988[19]. Furthermore, significant improvements in engraftment and overall survival have been achieved, including disease-free survival and reduced incidence of chronic graft-versus-host disease with CB compared with matched unrelated donor[208]. Another major disadvantage that remains with CB transplantation is the very slow engraftment kinetics of neutrophils and platelets.

Limiting cell loss during the processing of CBUs, cryopreservation and thawing of samples is critical since the starting cell content is far smaller compared with marrow and apheresis grafts[136]. The success of transplantation and engraftment is associated with both the number and the potency of cells transplanted[209]. Therefore, improving the cryopreservation of CB grafts may be of significant benefit for the outcome of the transplantation. The standard intracellular CPA for HSC grafts is DMSO[210,211]. However, patients can experience a wide range of side effects when infused with DMSO, including vomiting and, in rare cases, severe problems like breathing and vision issues and allergic

reactions. Numerous side-effects have been related with DMSO transfusion into patients involving nausea, headache, vomiting, hypertension[146]. Usually, DMSO has been demonstrated to have chemical toxic influences on cells causing damage of proliferative capability of HSPCs[138]. Consequently, because of these toxic effects some safety measures are necessary during cryopreservation procedure. Despite being used for 50 years, the short- and long-term impacts of DMSO on HSC function and cell quality remain poorly investigated, but a recent study revealed that very low concentrations of DMSO are sufficient to elicit epigenetic changes in human cells, leading to changes in gene expression[136,212]. But supplementation with new CPAs such as sugars can improve post thaw cell viability and potency and even reduce DMSO concentration requirements[191,213].

Recent advances in the field of cryobiology have led to the development of new commercial cryopreservation solutions. Although the exact makeup of these solutions is not fully disclosed, it is important to assess their usefulness in protecting stem cell grafts from cryoinjury. Such a CPA or solution should be safe for use in humans, support post thaw viability equal to or superior to DMSO and efficiently protect HSCs from cryoinjury to sustain long-term engraftment. To date, only PIM has been investigated in the context of PBSC grafts. In combination with albumin, PIM has been shown to provide similar recovery of CD34+ cells and CFUs compared with 10% DMSO[149]. Moreover, PBSC grafts cryopreserved with PIM have been shown to support similar long-term engraftment compared with DMSO controls[150]. Three other commercial DMSO-free cryopreservation solutions are currently available that could potentially be of use for HSC grafts. CSL is a xenogeneic- and serum-free medium formulated for the long-term storage of cells at -80°C or in liquid nitrogen. Successful cryopreservation of *ex vivo*-expanded CB T cells and nematode microfilaria[152] was recently reported with CSL. CN is manufactured entirely from naturally occurring CPAs. It has been shown to be superior to DMSO-containing

cryopreservation media when used with mesenchymal stromal cells[90]. Finally, CPP is a new DMSO- and serum-free balanced salt-based formulation that contains glycol derivatives with known cryoprotective abilities and molecular-grade, non-toxic protein components of xenogeneic origin with low immunogenic implications. In this study, we set out to define the impact of these four relatively new CPAs on CBUs, benchmarking them against a clinical-grade DMSO/dextran-40 cryopreservation solution as a control.

2.3. Materials and Methods

2.3.1. Cryopreservation solutions

Clinical-grade 5X DMSO (55% DMSO w/v in 5% w/v dextran-40) and CN were obtained from Akron Biotech (Boca Raton, FL, USA), CSL was obtained from DiagnoCine (Hackensack, NJ, USA), PIM was obtained from Pharmacosmos (Holbæk, Denmark) and CPP 5X was obtained from Ad Infinitum Cell Preservation Technologies (Plymouth,MI, USA).

2.3.2. CBU processing and cryopreservation

All CBUs (n=16) were obtained from the Canadian Blood Services CB for Research Program after obtaining institutional ethical approval (REB PROTOCOL REFERENCE # 2013.016 - Hematopoietic stem cells and transfusion medicine) and written informed consent from CB donors. Each CBU had a minimum volume of 50 mL and a TNC count greater than 0.9 $X10^9$ but inferior to 1.5 $X10^9$. Upon arrival, CBUs were processed to prepare the leukocyte rich plasma (i.e., buffy coat) by adding a solution consisting of 6% hetastarch in 0.9% sodium chloride (Hespan; B. Braun Medical Inc, Irvine, CA, USA) corresponding to 20% of the CBU volume. The mixture was gently agitated and then incubated at room temperature for 90 min. The buffy coat was collected and centrifuged at 300 X g for 10 min. The cell pellets were resuspended in plasma up to 10 mL kept at 4°C,

and a TNC count was performed using a pocH-100i hematology analyzer (Sysmex Corporation, Mississauga, Canada).

After centrifugation at 300g for 10min, the final cell pellets were resuspended in the different cryopreservation solutions (mean, 22.1 $X10^6$ cells/mL, range, 11.0-42.2 $X10^6$ cells/mL). Samples were frozen in a series of 1.2-mL cryovials using either an isopropyl alcohol-based device (n = 9) following the manufacturer's instructions (Mr. Frosty; Thermo Fisher Scientific, Waltham, MA, USA) or a CryoMed controlled-rate freezer (n = 7) (ramp 1.0°C/min until sample = -4.0°C, ramp 25.0°C/min until chamber = -40.0°C, ramp 10.0°C/min until chamber = -12.0°C, ramp 1.0°C/min until chamber = -40.0°C and ramp 10.0°C/min until chamber = -90.0°C) as indicated. All vials were then stored for a minimum of 24 h in a liquid nitrogen storage tank until use.

2.3.3. Thawing of CBUs

Buffy coat aliquots were thawed following a thaw and dilute procedure optimized for CBUs. In short, 1-mL aliquots were rapidly thawed in a 37°C water bath and then diluted 5-fold with a thaw solution consisting of 4% human albumin (Grifols Ltd, Barcelona, Spain) in Plasma-Lyte A (Baxter, Deerfield, IL, USA) in a two-step process that included two 15-min osmotic equilibration periods[214]. Thawed samples were then centrifuged at 300 X g for 10 min, and cells were resuspended in 2 mL of Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) with 2% fetal bovine serum (FBS) (HyClone; Thermo Fisher Scientific).

2.3.4. Flow cytometry analysis

An Attune fluorescence-activated cell sorting (FACS) flow cytometer (Thermo Fisher Scientific) was used to analyze CB, platelet, leukocyte, and BM preparations. Compensation was carried out with fluorescence-labeled microbeads (BD Biosciences, San

Jose, CA, USA) and fluorescence intensity minus one stained sample were used as a control to set quadrants and gates. Dead cells and debris were gated out by forward and side scatter or Sytox AADvanced staining (Thermo Fisher Scientific). For viability analysis, 1.0 $X10^6$ nucleated cells were treated with 2 mL ammonium chloride- based lysis solution (BD Biosciences) to lyse the red cells. Next, cells were pelleted (250 X g for 8 min) and suspended in annexin V binding buffer (Thermo Fisher Scientific) followed by staining with CD34-phycoerythrin (PE), CD45-allophycocyanin (APC), EpcR-APC, CD45RA-fluorescein isothiocyanate (FITC) and CD90+APC antibodies (BD Biosciences) and annexin V-Alexa Fluor 488 and Sytox AADvanced following the manufacturers's instructions. Samples were kept on ice after further dilution with annexin V binding buffer, and data were acquired within 1 h. Flow cytometry cell gating strategy for CD45+CD34+ cell events was done following the recommendations of the International Society for Hematotherapy and Graft Engineering (ISHAGE)[215], with the exception that viability of CD34+ cells was measured with annexin V as previously reported[214].

2.3.5. CD34+cell enumeration

Stem-Kit reagent (Beckman Coulter, Marseille, France) was used to measure absolute CD45+ and CD45+CD34+ cell counts following the manufacturer's recommendations. Absolute CD34+ cell count was determined based on the number of live CD34+ cell events in the ISHAGE CD34+ cell gate (a) and the number of events in the bead gate (b) according to the following formula:

$$\text{Absolute } CD34^{+} \text{cell } /\mu L = \frac{a \times \text{bead concentration (beads}/\mu L) \times \text{dilution factor}}{b}$$

2.3.6. CFU assay

CFU assays were performed by plating CB TNCs (50 000 per plate) in methylcellulose-based media (MethoCult H4434; STEMCELL Technologies, Vancouver, Canada) in duplicate and incubating for 2 weeks in a humidified atmosphere (5% carbon dioxide) at 37°C. Colonies were scored by microscope based on standard morphological criteria: CFU total, BFU-E, CFU-GM and CFU-GEMM progenitors.

2.3.7. Freezing of CB CD34+-enriched cells and cell expansion cultures

CB CD34+ cells were enriched from freshly processed CBUs using the EasySep Human CD34 Positive Selection Kit II (STEMCELL Technologies) following the manufacturer's instructions (purity >90%). CD34+ cells were frozen in aliquots in the presence of DMSO (10% DMSO/40% FBS/ 50% IMDM) or CPP-STEM (CPP-STEM/40% FBS/40% IMDM). The potency of the CB CD34+ cells were tested using a cell suspension expansion assay. CD34+ cells (starting density of 12 000 cells/mL) were placed in culture in serum-free expansion medium (STEMCELL Technologies) with stem cell factor 20 ng/mL, thrombopoietin 20 ng/mL and FLT-3 ligand 20 ng/mL (PeproTech, Rocky Hill, NJ, USA), StemRegenin1 at 750 nM, UM171 at 35 nM and low-density lipoprotein 5 mg/mL (STEMCELL Technologies) and 1% penicillin-streptomycin (Thermo Fisher Scientific) for 14 days.

2.3.8. Xenotransplant model

Eight- to 10-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) (The Jackson Laboratory, Bar Harbor, ME, USA) mice were irradiated at 300 cGy with 137Cs (Gammacell 40 Exactor; Best Theratronics Ltd, Ottawa, Canada) and transplanted intravenously with CB TNCs treated with OKT3 (Bio X Cell, Lebanon, NH, USA)[163]. Cell dose was 1.6 $X10^6$ cells/mice. Human platelet engraftment analyses in xenotransplants were done at 4 weeks (short-term), 10 weeks (mid-term) and >14 weeks (long-term) after

transplantation[216]. Human platelet levels were measured following a single-platform protocol. In brief, 5 mL of mouse blood was diluted 10 times with phosphate-buffered saline and stained for 20 min at 4°C with 2 mL of human PE-conjugated CD41a (BD Biosciences). After incubation, red cells were lysed by treatment with 250 mL of 1X BD Pharm Lyse solution (BD Biosciences) at room temperature for 15 min. Samples were then diluted with 250 mL of phosphate-buffered saline followed by the addition of 50 mL of

AccuCheck Counting Beads (Thermo Fisher Scientific). A fixed volume of 450 mL per sample was acquired by FACS. For leukocytes, mouse blood was stained with human anti-CD45-APC, CD33-PE and CD19-FITC and then red cells were lysed with 1£ BD Pharm Lyse solution (BD Biosciences), after which samples were analyzed by FACS. BM engraftment analysis was performed after 22 weeks. BM cells were harvested from hind legs, red blood cells were lysed with ammonium chloride (STEMCELL Technologies) and BM cells were stained with the following antibodies: CD19-PE, CD14-PE, CD45-APC, CD33-PE, CD34-PE, CD3-APC human CD41a (GPIIb)-FITC, CD56-PE and CD45-FITC (BD Biosciences).

2.3.9. Statistical analyses

Statistical analyses were performed using the statistical software Prism 8 (GraphPad Software, San Diego, CA, USA). Paired t-test, two tailed analyses of variance or mixed model analysis were employed for in vitro data analysis. *In vivo* engraftment data were analyzed using the Mann-Whitney test. $P < 0.05$ was considered significant. Data are presented as mean± standard error of the mean unless otherwise stated.

2.4. Results

2.4.1. Impact of different CPAs on viability and potency of CB stem cell grafts post thaw

Since the speed of engraftment is largely dictated by the dose of cells transplanted and their potency[217], the DMSO-free freezing media was first evaluated to understand if it provided adequate cryoprotection of CB HSC grafts and post thaw cell recovery and potency. CBUs were processed for volume and red cell reduction as commonly done in the CB banking industry, after which the buffy coats were split and frozen in equal parts with either clinical-grade DMSO/dextran-40 cryopreservation solution (i.e., DMSO control) or the DMSO-free cryopreservation solutions CPP, CSL, CN and PIM.

The net recovery of TNCs post thaw was found to be similar with all five cryopreservation solutions tested: 81± 4% with DMSO, 85±3% with CPP, 80±6% with CSL, 80± 6% with CN and 88± 1% with PIM (P> 0.05). The capacity of the different cryopreservation solutions to protect CB cells from osmotic shock and ice crystal damage leading to loss of cell membrane integrity and cell viability was investigated by performing annexin V-based viability analysis (Figure 6). The highest post thaw viability of CD45+ cells was achieved with CPP (74±3%) (Figure 6A). CSL (58±2%) and PIM (50±3%) provided equal post thaw viability that was, however, inferior to that seen with CPP or DMSO (70 ±5%), whereas CN (8±1%) provided the lowest viability. The low viability seen with CN was due to loss of membrane integrity in CB cells post thaw, as evidenced by the high percentage of necrotic cells (mean-86.8%). The proportion of apoptotic CD45+ cells was similar between CPP, CSL and DMSO, whereas the lowest proportion of necrotic CD45+ cells was seen with CPP and DMSO. By contrast, CPP (80±3%) and PIM (86±3%) provided the highest viability for CD34+ cells (Figure 6B), whereas DMSO (75±3%) and CSL (75±4%) provided equivalent results and CN the lowest (69± 5%).

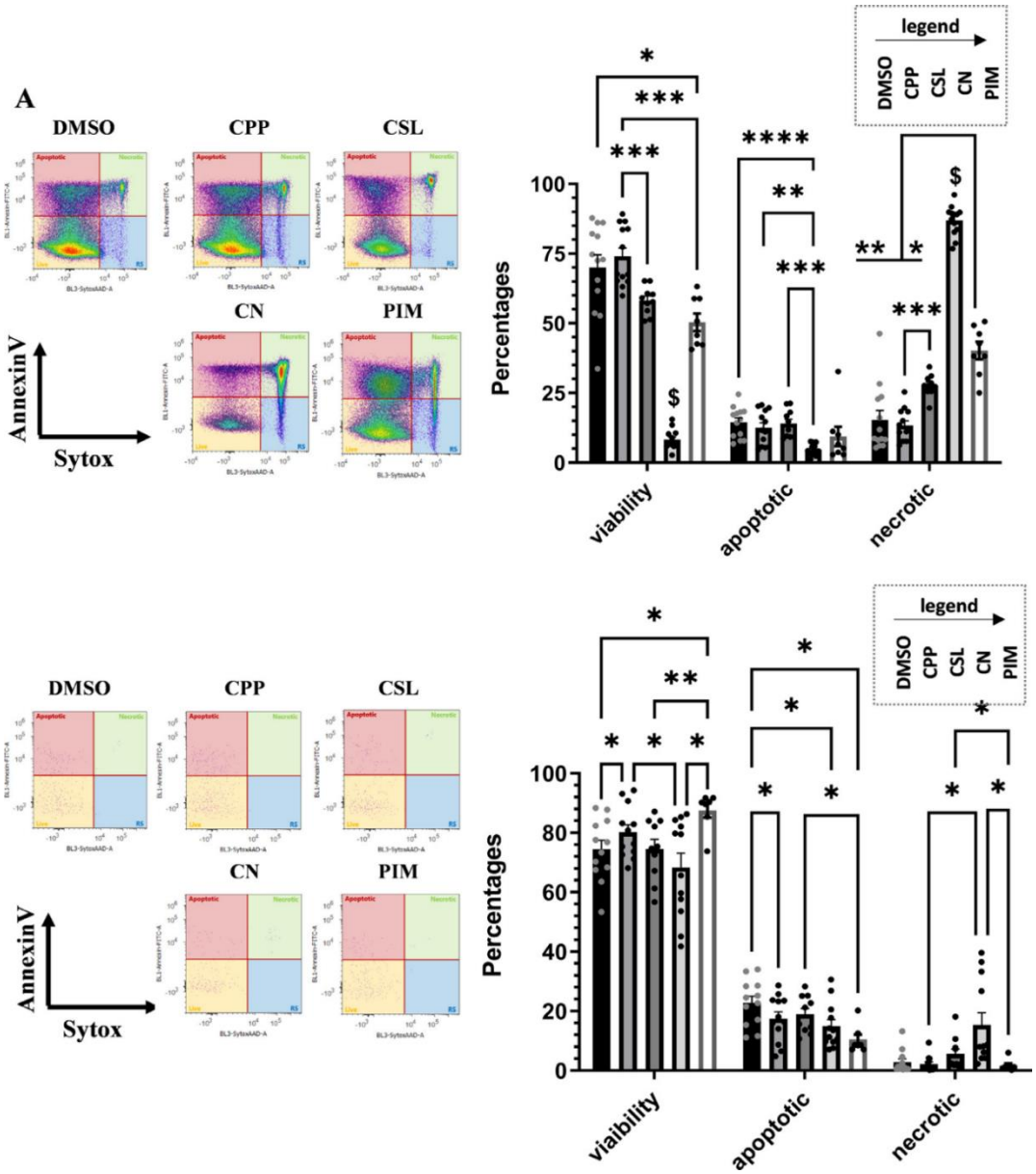

Figure 6: Cryoprotective properties of DMSO-free cryopreservation solutions on CB HSCs.

(A) Representative flow cytometry of CD45+ cell annexin V/Sytox viability analysis of CB cells frozen with indicated freezing media. (B) Proportion of viable, apoptotic, and necrotic CD45+ CB cells. (C) Representative flow cytometry of CD34+ cell viability analysis from CBUs frozen with different CPAs. (D) Proportion of viable, apoptotic, and necrotic CD34+ cells. Viability measured with annexin V/Sytox dye. Data represent mean § SEM (n = 8-13). Two-way ANOVA with Greenhouse_Geisser correction and Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; $P < 0.0001 CN versus any other condition. ANOVA, analysis of variance; SEM, standard error of the mean.

Next, the net number of viable CB cells recovered post thaw was determined with the single ISHAGE platform to accurately measure the number of viable CD45+ and CD34+ cell counts post thaw (Figure 7). This set of results confirmed most of the previous observations. The viability and mean number of viable CD45+ cells were highest in the

CPP and DMSO samples (Figure 7A, B). CN and PIM provided the lowest recovery of viable CD45+ cells, whereas CSL provided an intermediate response. Regarding CD34+ cells, their viability was similar with all cryopreservation solutions, except for CN, which demonstrated the lowest viability (Figure 7C). However, the net recovery of viable CD34+ cells post thaw was superior with CPP (mean, 97%) and DMSO (arbitrarily set at 100%) followed by CSL (mean, 67%), PIM (mean, 33%) and CN (mean, 17%) and like that previously noted with CD45+ cells (Figure 7D). Similar observations were made when the net number of viable annexin V/Sytox double negative CD45+ and CD34+ cells was estimated.

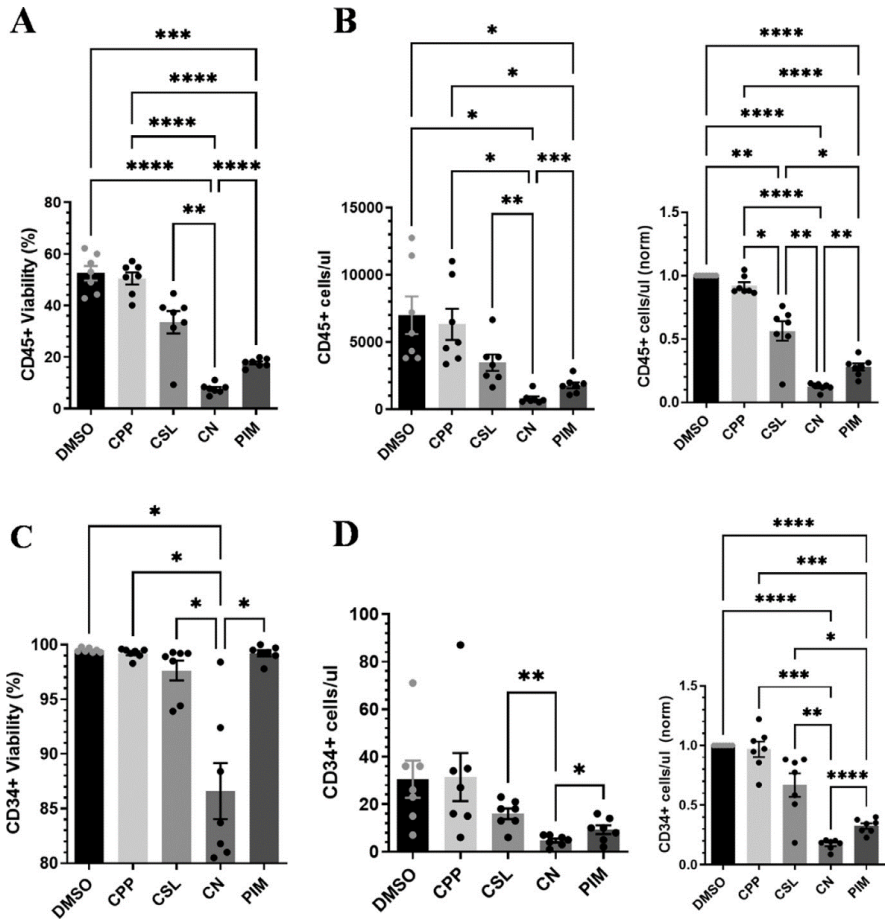

Figure 7: Impact of DMSO-free Cryosolutions on the recovery of viable CB HSCs post thaw.

(A) Viability of CD45+ cells measured following ISHAGE gating strategy guidelines with 7-AAD DNA staining. (B) Net number of viable CD45+ cells presented as cells/mL (left graph) or as norm to that measured in the DMSO control (right graph). (C) Viability of CD34+ cells measured following ISHAGE gating strategy guidelines with 7-AAD DNA staining. (D) Recovery of viable CD34+ cells presented as cells/mL (left graph) or as norm to that measured in the DMSO control (right graph). One-way ANOVA with Greenhouse Geisser correction and Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Norm, normalized; 7-AAD, 7-aminoactinomycin D.

Finally, the CFU assay was used to measure the potency and recovery of progenitor's post thaw. CFU is the most common potency assay used to track the engraftment potential of HSC grafts. The number of progenitors detected per cell plate (i.e., cloning efficiency) is presented in Figure 8A. The type and distribution of myeloid progenitors detected were similar between all cryopreservation solutions tested, though the frequency varied significantly.

More importantly, the net number of CFUs recovered for CBU samples cryopreserved with CPP (11 569±3251) was comparable to that seen with the DMSO control (11 792±2691) (Figure 8B). CSL provided the next best recovery (7224 ±1231), whereas CN (2126±477) and PIM (5737±1167) provided potencies inferior to DMSO and CPP. This confirmed that CN and PIM are not as effective for the cryopreservation of CB hematopoietic progenitors under current testing conditions.

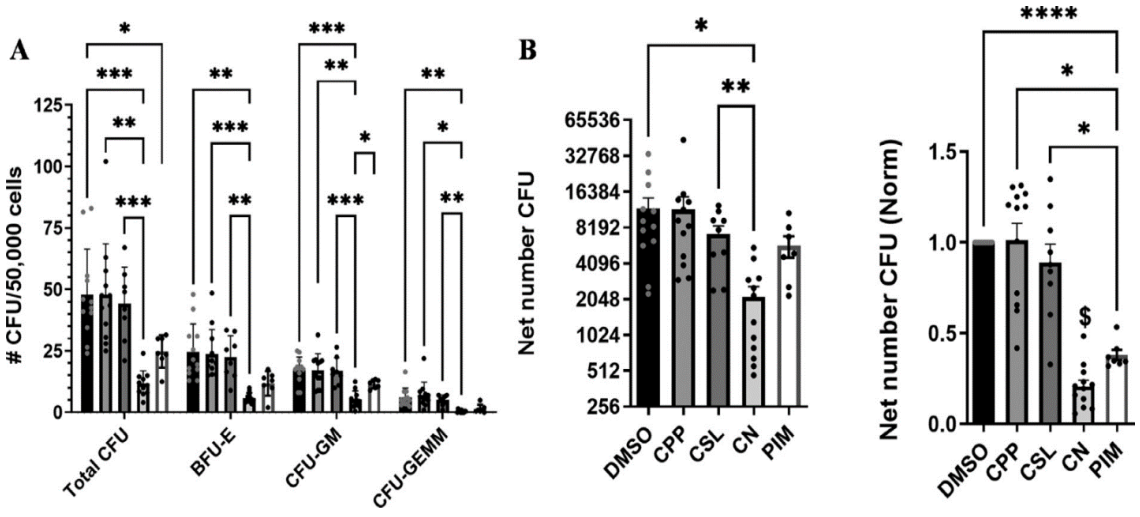

Figure 8: Impact of DMSO-free Cryosolutions on potency of CB progenitors post thaw.

(A) Number of CFUs detected per 50 000 TNCs plated. CFU total, BFU-E, CFU-GM and CFU-GEMM progenitors were presented. (B) Net number of CFUs recovered post thaw. Normalized number of CFU total presented in right graph (data normalized to DMSO control). Data represents mean±SEM (n = 7-12). Mixed model analysis with Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; P < 0.01 versus any other condition. BFU-E, burst-forming unit_erythroid; CFU-GEMM, CFU-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM, CFU-granulocyte-macrophage; SEM, standard error of the mean.

Taken together, these results revealed that not all DMSO-free cryopreservation solutions can be used in their current formulation with CB cells when combined with slow cooling rates. However, the results also showed that cryopreservation with CPP provided suitable post thaw cell viability, recovery, and progenitor maintenance. Hence, the

remainder of the study focused on investigating the cryoprotective properties of CPP on CB HSC and progenitors.

2.4.2. Impact of CPAs on viability and recovery of HSPCs expansion

A series of experiments were designed to focus on the impact of CPP on HSC and progenitor cells. To overcome the very low frequency of HSCs (<0.6% of CB TNCs), CB CD34+ cells were first enriched and then frozen with 40% FBS and 40% IMDM supplemented with either 10% DMSO or CPP. Viability analyses of the CD34 +-enriched cells post thaw confirmed the original observation that CPP efficiently protects CB CD34+ cells during cryopreservation, as the proportion and net number of viable CD34+ cells were similar or greater to that observed with DMSO (n = 3) (Figure 9A, B). Moreover, the viability of the CD34+CD90+ cell subset further enriched in HSCs was excellent with CPP compared with the DMSO control (Figure 9C), resulting in a high number of CD34+CD90+ cells recovered after thaw (Figure 9D).

CB HSCs have a very high proliferation capacity, and several small molecules, including UM171[32] and StemRegenin1[180], have been identified that further promote expansion of HSCs over differentiation. Hence, the growth capacity of the CB stem and progenitor cells was tested by culturing CD34+ cells with early-acting cytokines and with UM171 and StemRegenin1 supplementation to support substantial expansion of stem and progenitor cells (n = 3) (Figure 9E)[32]. The overall level of TNC and CD34+ cell expansion achieved in CPP CD34+ cultures was almost identical to that observed with the DMSO control (n = 3) (Figure 11F, G). The expansion of HSC-enriched cells was investigated by tracking the CD34+CD45RA- and CD34+CD45RA-Epcr- High subsets progressively enriched in HSCs (Figure 9E)[32]. Expansion in CPP cultures of both HSC-enriched subsets at all time points was also like that seen in the control DMSO cultures. For instance, the expansion of CD34+CD45RA-cells on day 14 was 270±75 versus 253±66 for DMSO and

CPP, respectively. Hence, these results suggest that HSCs and progenitors with high proliferative capacity are well preserved by CPP (Figure 9H, I).

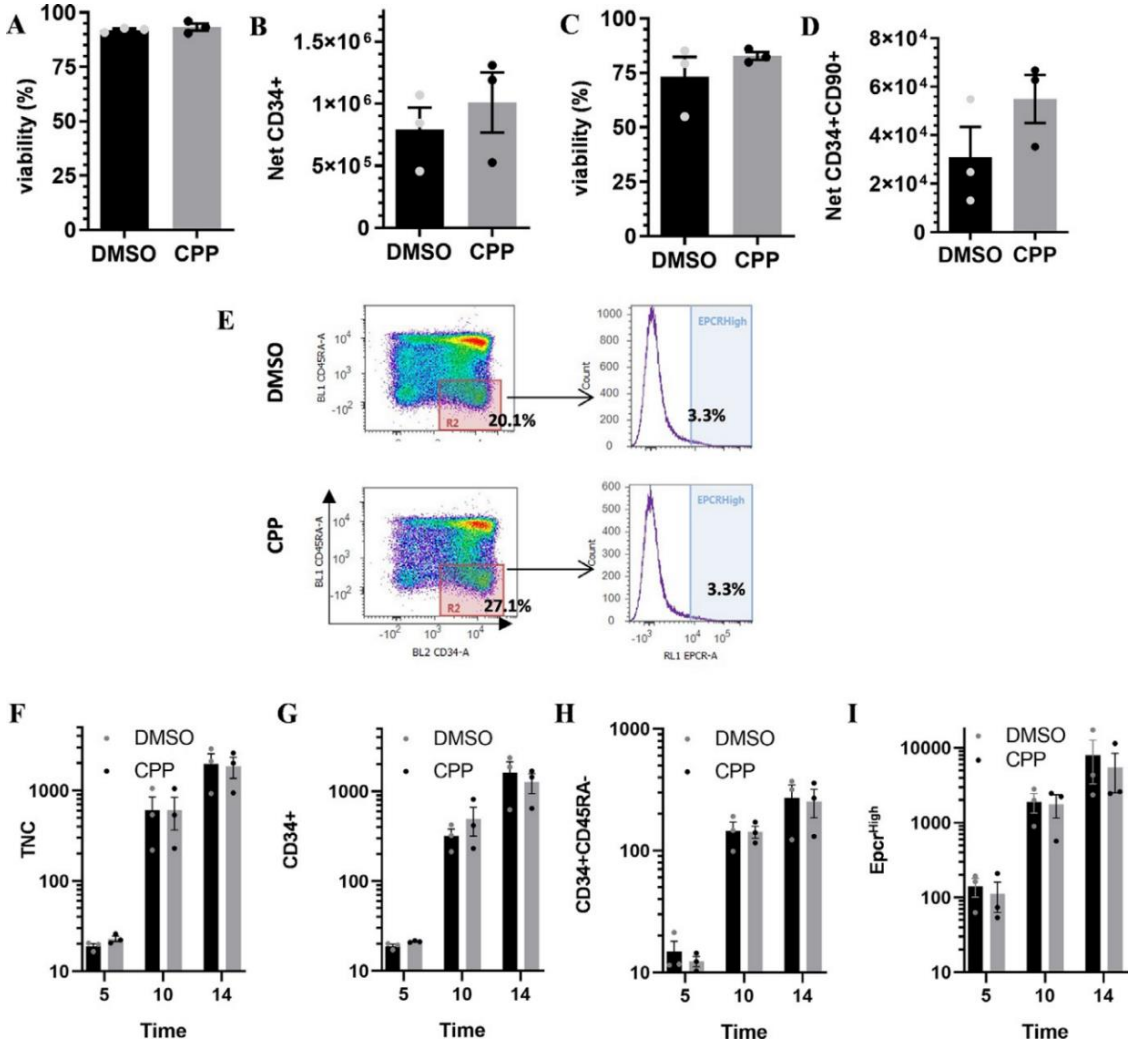

Figure 9: Viability and expansion potential of HSPCs cryopreserved with CPP.

(A, B) Viability and net number of viable CD34+ cells post thaw. Results are based on annexin V/Sytox AADvanced staining and from the thawing of CD34+-enriched cells frozen with indicated cryopreservation solution (n = 3, mean± SD, t-test, no significant differences). (C, D) Viability and net number of viable CD34+CD90+ cells post thaw. Data represents mean± SEM (n = 3, t-test, no significant differences). (E) Representative flow cytometry analysis of cell surface antigens CD34, CD45RA and EpcR at day 14 in stem cell expansion cultures established with CD34+ cells frozen with DMSO or CPP. Proportion of EpcrHigh cells within the CD34+CD45RA- subset presented in histogram. Data represents mean± SEM (n = 3, t-test, no significant differences). (F-I) Fold expansion of TNCs and CD34+, CD34+CD45RA- and EpcrHigh cells in culture at indicated time

point. (F) Data represent mean± SEM (n = 3, t-test, no significant differences). EpcrHigh, CD34 +CD45RA-EpcrHigh; SD, standard deviation; SEM, standard error of the mean.

2.4.3. Effect of CPAs on engraftment activity of HSC graft by functional validation through xenotransplantation assays

The previous results demonstrated that CPP provides very good cryoprotection to both mature CD45+ cells and CD34+ stem and progenitor cells, which was confirmed with two independent potency assays on independent biological samples. However, only transplant experiments can confirm whether HSC functions are well preserved. To this end, two xenotransplantation assays were performed to compare the engraftment activities of CB TNCs (i.e., CB buffy coat cells) cryopreserved with CPP or control DMSO. Unwashed diluted single CB cells were transplanted into irradiated NSG mice (six to seven mice/arm, two independent CBUs tested). This model was selected, as it closely mimics current clinical practice except for the OKT3 T-cell antibody pre-treatment used to prevent graft-versus-host disease in mice[163].

Engraftment analyses were first done by tracking human cells in the periphery at week 4, week 10 and week 22. Human platelets were readily detected in both mouse groups (Figure 10A), with CPP recipients showing high human platelet levels at all three time points. Likewise, the levels of human CD45+ chimerism in the periphery of CPP and DMSO graft recipients were similar (Figure 10B). Moreover, both CD33+ myeloid leukocytes and CD19-B cells were seen in both mouse groups.

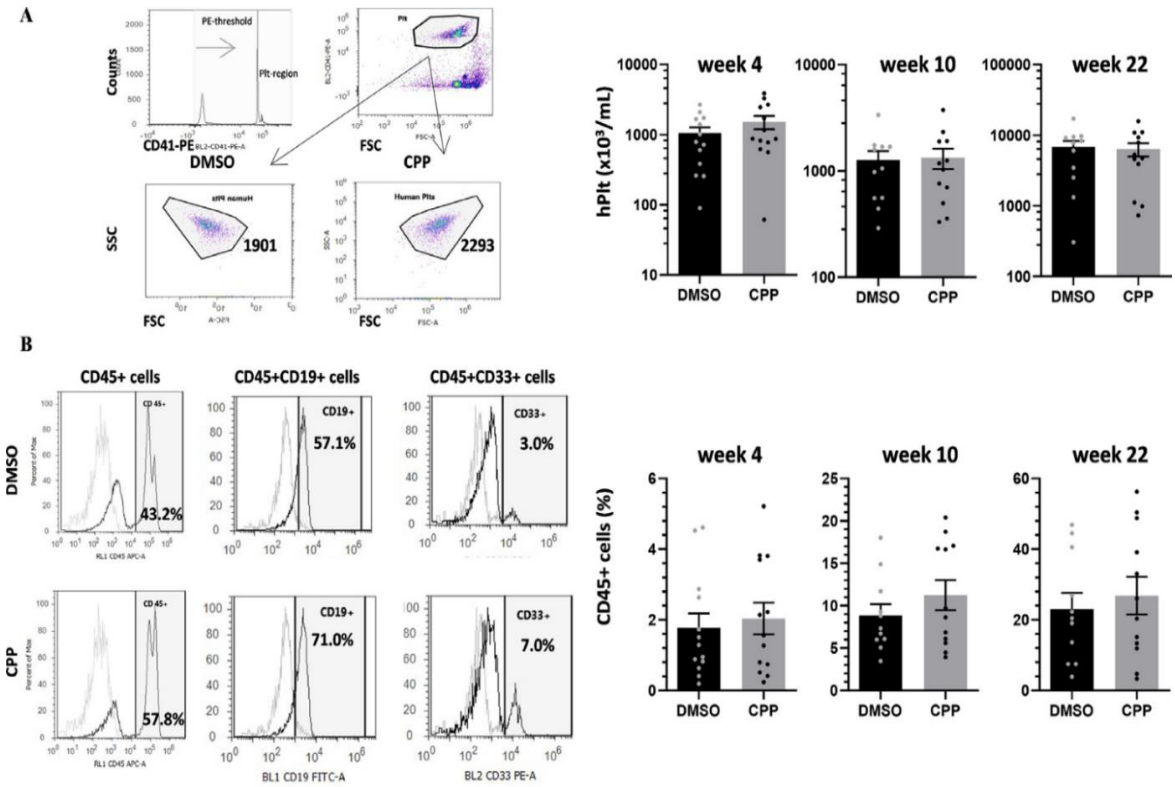

Figure 10: Levels of human platelets on human leucocytes in mice transplanted with DMSO-free cryopreserved HSCs and progenitors.

(A) Representative flow cytometry of human platelets in murine blood in DMSO and CPP recipients. Mean levels of human platelets at indicated time point in DMSO and CPP recipients presented on the right. Mean ± SEM presented in histograms. Each symbol represents the value of an individual mouse, with six to seven mice per group per CBU tested (n = 2, Mann-Whitney, no significant differences). (B) Representative flow cytometry of human leukocytes in murine blood in DMSO and CPP recipients. Mean frequency of human CD45+ leukocytes in murine blood leukocytes at indicated time point in DMSO and CPP recipients presented on the right. Each symbol represents the value of an individual mouse, with six to seven mice per group per CBU tested (n = 2, Mann-Whitney, no significant differences). SEM, standard error of the mean.

Long-term BM engraftment analysis was carried out 22 weeks post-transplantation, as earlier investigation can be compromised by the contribution of short-term HSCs. Human chimerism analysis revealed that the average level of human chimerism between the DMSO (58±4%) and CPP (54±4%) groups was similar for both CBUs tested (Figure 11A, B). Next, multi-lineage differentiation analysis of the human BM cells was carried out using various cell surface markers. Both mouse groups had equivalent

frequencies of CD34+ progenitors, CD34+CD38-HSC-enriched cells and CD34+CD38+ cells (Figure 11C).

Likewise, CD19+ B cells, CD3+ T cells, myeloid CD33+ cells and CD14+ monocytes were all detected (Figure 11D). Together, these results provided indications that stem and progenitor cells with short- and long-term engraftment activity are adequately protected from cryoinjury by CPP.

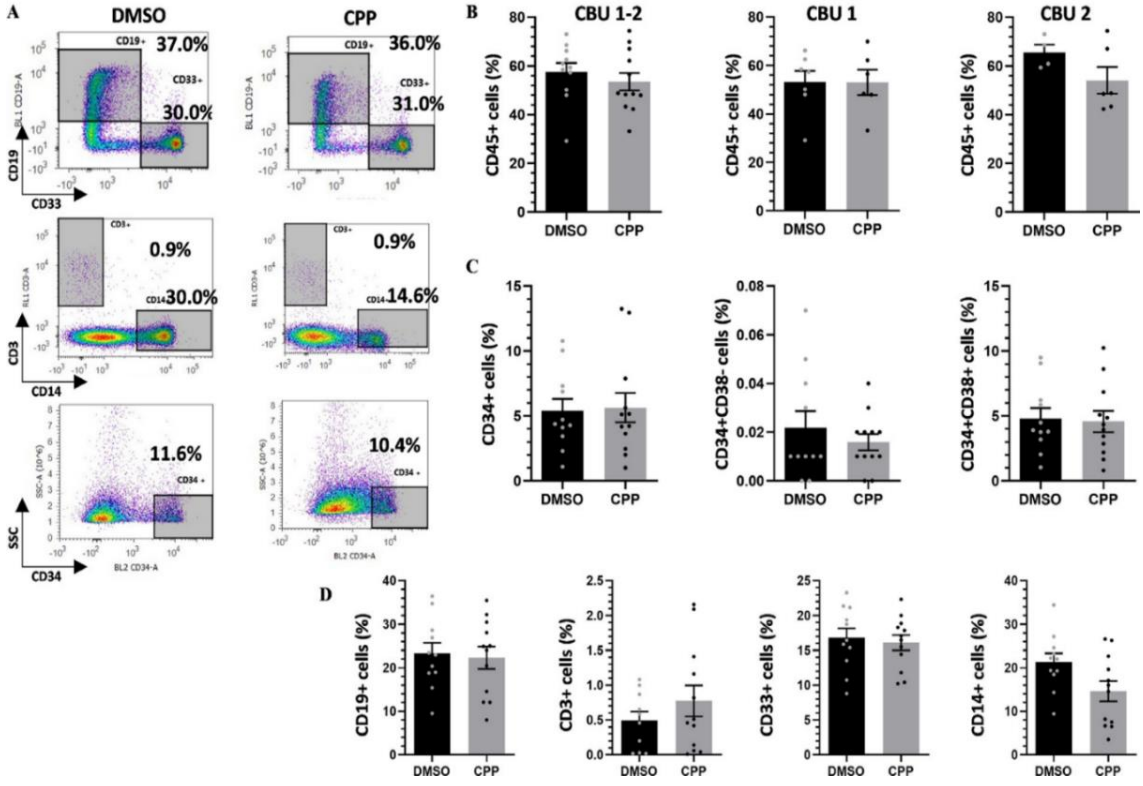

Figure 11: Long-term human BM engraftment in humanized mice.

(A) Representative BM analysis of BM extracted from DMSO and CPP recipients using different antibody panels. (B) Human CD45+ BM chimerism in humanized mice 22 weeks post-transplantation. Mean of the two CBUs tested (left graph) and results of individual CBU 1 and CBU 2 analyses (middle and right). (C, D) Mean frequency of indicated human BM subpopulations in DMSO and CPP mouse transplant groups. Mean ± SEM for six to seven mice/ group/CBU (n = 2, Mann-Whitney, no significant differences). SEM, standard error of the mean.

2.5. Discussion

Cryopreservation is the best method of storing stem cell grafts until use, especially regarding CBUs, for which the storage duration is typically unknown. A large body of research has identified new CPAs, including sugars, polymers, amines, and ice recrystallization inhibitors, that may soon change the future of cryopreservation[127,191]. In this study, my colleagues and I investigated whether new DMSO freezing media could replace DMSO and provide adequate cryoprotection to HSC grafts. To this end, four different DMSO-free cryopreservation solutions were compared, and the results revealed that CPP provided the best recovery of viable CD45+ and CD34+ cells post thaw, followed closely by CSL. CPP was also shown to retain high potency and engraftment activity of CB HSCs and progenitors, making it a CPA of interest for CB HSCs and grafts.

CPAs are crucial to limit irrevocable damage to cells during freezing and thawing that would otherwise reduce viability and function[61]. The majority of CPAs act by interacting one way or another with water molecules, thereby altering phase transition or stabilizing cell membranes, proteins, and organelles[152]. DMSO is the standard intracellular CPA used for HSC grafts[85]. It permeates cell membranes and protects cells from cryoinjury associated with slow cooling rates by preserving cell volumes and reducing "solution effects." The identification of novel intracellular and extracellular CPAs has led to the development of new cryopreservation protocols. Many studies have focused on supplementing DMSO with new extracellular CPAs such as trehalose or other sugars to reduce the concentration of DMSO in HSC grafts to as little as 2%[94,112,123]. Importantly, the reduction in DMSO concentration has reduced the frequency of infusion-related adverse events. CryoStor is a commercially available solution that can be used to freeze CBUs at lower DMSO concentrations[85].

However, DMSO-free cryopreservation solutions are slowly emerging as new alternatives. Here four such freezing media were compared. CB cells cryopreserved with CPP provided results superior or comparable to those seen with the DMSO/dextran control regarding the net number of viable ISHAGE CD45+ and CD34+ cell counts. CPP and DMSO also provided the best protection against cryoinjury-mediated loss in membrane integrity post thaw. In addition, CPP often outperformed other DMSO-free freezing media. Indeed, viability analyses revealed that CPP protected CB CD45+ and CD34+ cells very well. These results were confirmed when the net number of annexin V/Sytox-viable cells was calculated and found to be superior in aliquots frozen with CPP. CSL was the second best of the DMSO-free cryopreservation solutions, as it provided very good post thaw CB cell viability and potency, often matching that of CPP and DMSO. However, the net number of viable CD45+ cells was slightly inferior ($P < 0.05$) with CSL compared with CPP and DMSO. Moreover, the recovery of CD34+ cells was slightly reduced, although the differences were not significant. The lowest recovery of viable cells and lowest potency were seen with CN, which was the direct result of a high level of necrosis. The most plausible explanation for this result is the excessive formation of intracellular ice due to insufficient cell dehydration during cooling and freezing, leading to loss of membrane integrity post thaw. CN was originally derived from mesenchymal stromal cells, which differ in many ways from HSCs. Further studies are needed to investigate the poor performance of CN on HSC cryopreservation.

The capacity of the different freezing media to retain the potency of hematopoietic grafts was investigated using the CFU assay, a 2-week growth culture assay that can detect further loss of viability and function due to delayed-onset apoptosis. These results aligned very well with the ISHAGE and annexin V/Sytox data sets, as the three leading freezing media were CPP and DMSO followed by CSL. Surprisingly, PIM supplemented with 2%

human albumin did not match the recovery of viable CD45+ and CD34+ cells or potency seen with the DMSO control, which was expected based on previous reports[149,218]. These discrepancies could be due to differences in experimental setup or cell type, as the original studies were done with adult PBSCs. However, recent studies lend support to the results reported herein. Indeed, a recent study from the same team ruled out PIM as a sole CPA for adipose-derived stromal/stem cells but showed that combining it with a low concentration of DMSO (1-2%) improved the viability and function of these cells post thaw[149].

These findings were recently confirmed with T cells[94]. The annexin V/Sytox viability results presented here suggest that as a stand-alone CPA with albumin, PIM fails to appropriately protect HSCs from cryoinjury, leading to cell membrane rupture, likely due to excessive intracellular ice formation. The results with supplementation of PIM with the permeating CPA DMSO support this suggestion. The combination of extracellular and intracellular CPAs is certainly a common strategy used to optimize cryopreservation protocols[90]. Originally, CPP was reported to be a good cryopreservation solution for adherent cells (www.aicryo.com), but this did not guarantee that it would be equally suitable for HSCs. Indeed, differences in membrane composition, resulting in different types of resistance to changes in osmotic pressure and permeability to water and solutes, can lead to different post thaw outcomes[61]. CPP does not contain any DMSO or sulfoxide compounds. The balanced salt-based formulation contains glycol derivatives, with individual concentrations conforming to Good Manufacturing Practice standards, as indicated by the US Food and Drug Administration's Code of Federal Regulations Title 21. The serum-free formulation contains non-toxic polymeric protein components. The specific formulation is regarded as a trade secret by the manufacturer. Given the positive

outcomes achieved with CPP, the authors carried out additional assays to further investigate its use and potential.

The great majority of research done on HSCs and clinical-based HSC expansion protocols is dependent on the pre-isolation of stem and progenitor cells by cell enrichment (e.g., CD34 selection)[85]. As previously observed with CB buffy coats, CPP supported very high post thaw viability of CD34+-enriched cells. Similar high viability was seen for the CD34 +CD90+ subset further enriched in HSC activity. The DMSO and CPP frozen CD34-enriched cells were then tested in a liquid-based HSC expansion assay. The growth and expansion of CD34+ subsets progressively enriched in HSC activity (i.e., CD34+CD45RA- and CD34+CD45RA-EpcrHigh[213]) were found to be essentially similar between CPP and DMSO-cryopreserved CD34+ cells.

The engraftment activity of CPP-cryopreserved CB buffy coat grafts investigated using the NSG mouse transplant model. The results of the two transplant experiments confirmed that cryopreservation of CB cells with CPP preserved the engraftment activity of CB HSCs and progenitors. This was evident by the high levels of human platelets and lymphomyeloid leukocytes throughout the follow-up period and by the high level of long-term human BM engraftment in CPP recipients. Finally, normal multi-lineage HSC differentiation in BM was confirmed in both groups. These results agree with the potency assays but need to be interpreted with caution since only two CBUs were tested. Future work will need to extend these findings by testing additional donors and performing secondary transplants to provide assurance that long-term HSCs are adequately protected. Nonetheless, these results provide strong indications that HSCs and progenitors are properly protected from cryoinjury by CPP.

One limitation of the present work is that optimal cooling rates for the different freezing media have not been found and require future investigation. Hence, it is possible

that better results could be obtained with different freezing kinetics. However, the devices used here (passive cooling with Mr. frosty apparatus and controlled-rate freezer) are the most used in the areas of research and development with HSCs. This study also raises many important questions that need to be addressed in the future. For example, additional work is needed to investigate whether CPP can be translated from cryovials to the cryopreservation of whole CBUs (i.e., 25-mL product with greater cell density) or adult stem cell products whose cell density is far greater. Also, results obtained with CSL were encouraging and warrant further investigation as a second legitimate DMSO-free alternative. Finally, it remains to be seen whether supplementing PIM with a low concentration of DSMO can overcome the loss in cell recovery and potency observed here with CB cells.

2.6. Acknowledgements

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CHAPTER 3

IMPACT OF CRYOPROTECTANTS ON MITOCHONDRIAL RESPIRATION, GLYCOLYSIS AND CELL MEMBRANE INTEGRITY OF HSPCs

3.1. Summary

Background and aims: Current scientific studies suggest that cryopreservation affects the bioenergetic pathways and energy balance of cells which may lead to cell damage and cell loss. Therefore, it is paramount to understand the impact of CPAs used in cryopreservation on various metabolic processes. Additionally, as necrosis is the major reason of cell loss during cryopreservation, careful analysis should also explore the impact of CPAs on cell membrane.

Methods: An Agilent Seahorse XF analyzer was used to measure real time OCR in mitochondrial respiration and ECAR in HSPCs[1]. A lactate dehydrogenase (LDH) colorimetric assay was performed to measure membrane integrity and leakiness. A Lipid Peroxidation (LP) assay was used to measure the oxidative levels of lipid in cell membrane of HSPCs.

Results: Spare respiratory capacity (SRC) and maximal respiratory capacity were significantly greater in HSPCs previously cryopreserved with DMSO when compared to CPP. Gene expression analysis confirmed that DMSO cryopreserved HSPCs had the highest expression of glycolytic genes than HSPC cryopreserved with CPP or CSL. The LDH and LP results demonstrated that the short-term impact of the CPAs on cellular

[1] Seahorse experimental work was conducted in collaboration with Dr. Mary Ellen-Harper's lab, Department of Biochemistry, University of Ottawa. aided by Claire Fong McMaster, master's student in this lab.

membrane integrity and lipid peroxidation levels were similar and comparable to untreated cells.

Conclusions: My results demonstrate that the type of CPA used during cryopreservation can impact the bioenergetic pathways of HSPCs post thaw. In contrast, all CPA tested showed similar modest impact on cell membrane pre freeze suggesting that those tested are well tolerated.

3.2. Introduction

Cryopreservation is a standard practice worldwide owing to its successful application to many cell types and the high levels of cellular recovery that appear to be maintained on thawing. However, the negative impact of the cryopreservation process and that CPAs have on the quality and function of cells should be characterized. In Chapter 2, I tested a series of new CPAs and studied their efficacy in cryopreserved CB HSPCs. In this Chapter, I set out to test the how these CPAs influence the bioenergetic pathways and cell membrane of HSPCs.

Previous literature has shown that the HSC metabolism controls hematopoiesis, however the processes that connect metabolism to functionality of HSC remain very poorly understood[74]. Stem cells can undergo different types of metabolic alteration throughout their process of proliferation and quiescence cycle[77]. Lower mitochondrial content in HSCs, and the fact that HSCs shifts cells to a glycolytic metabolism indicate that quiescent HSCs use glycolysis instead of oxidative phosphorylation to attain energy necessities[154]. Mitochondria has recently emerged as critical not only for HSC differentiation and commitment but also for HSC homeostasis[156,165]. A recent research report revealed the there is significant loss of the mitochondrial complex III subunit Rieske

iron sulfur protein in fetal mouse HSCs which permits their proliferation but damages their ability to differentiate, ensuing prenatal death[184,219].

Recent evidence suggests that mitochondria could be involved in HSC fate and self-renewal potential[188]. A study showed that when mitochondrial activity of HSCs is lowered by chemically uncoupling the electron transport chain, it drives self-renewal under culture conditions that normally induce rapid differentiation[35]. Moreover, accumulating evidence indicates that mitochondria have been identified as the HSC's principal source of bioenergetics during process of differentiation. This phenomenon was shown by various genetic excision models; for example, as monitored in *bid*, *mtchII* and *tsc1*, in which mitochondrial function impairment was linked with prolonged expansion of the hematopoietic progenitor population[184].

Similarly, a study conducted by Guo *et al.*, found a novel target for expansion of UCB providing an enhanced understanding into the molecular regulatory networks linked with HSC metabolism. The authors reported that FBP1, a gene encoding a negative regulator of glycolysis, was greatly downregulated by GW9662, which is a potent antagonist of peroxisome proliferator-activated receptor gamma (PPARγ)[166]. This implied a connection between glycolysis and HSC expansion. Hence, several studies have established the importance of glycolysis in HSC biology, making it essential to investigate the role of bioenergetic pathways in HSCs during cryopreservation[154] .

Cryopreservation can also alter the metabolism of the cells, which can affect their function and differentiation potential[220]. A study compared the mitochondrial function and metabolic profile of fresh and cryopreserved human BM-MSCs using flow cytometry and mass spectrometry[123]. The results showed that cryopreservation reduced the mitochondrial membrane potential and decreased the glycolytic rate of MSCs[132]. Various studies have been undertaken over the past years on cryopreservation of mitochondria for fundamental

research into the nature of freezing damage[156]. Also, the studies from Fishbein's group suggested that, while rapid cooling in the presence of DMSO resulted in good preservation of ultrastructure and enzymatic profiles of rat liver mitochondria, coupled respiration was destroyed[221]. A bioinformatics analysis on sperm stem cells discovered that metabolic pathways play a significant role in cryopreservation, involving mechanisms of glycolysis or gluconeogenesis and TCA cycle[154] . Altogether, there are good deal of evidence that bioenergetic pathways are impacted by cryopreservation, however, how different CPAs may impact bioenergetics is less understood[166,188]. Therefore, one of the aims in this chapter was to investigate the impact selected CPAs have on these the mitochondrial and glycolytic pathways using CB HPSCs.

Cryopreservation through osmotic stress and ice recrystallization causes physical damage to the cells including the cell membrane, which induce cell death mostly through necrosis and to a lesser extent by apoptosis[182]. Cryobiologists constantly aim to lower cell losses during cryopreservation to augment the therapeutic efficacy of HSCs. Additional work is required to verify the site of cryoinjuries lead to cell death during freezing and thawing[222,223]. Early studies confirmed that there is membrane damage during cryopreservation but whether this is because of the nature of CPA or not, has been poorly understood[42,145]. Loss in membrane integrity leads to necrosis, which is the main mechanism in loss of viability during the freezing and thawing cycle[162]. A study by Guttridge, *et al.,* also reported that the post- thaw viability of CD34+ cells reduced as a function of storage time because of the heightened number of apoptotic and necrotic CD34+ cells, which was due to negative impact on cell membrane[75,217]. Latest developments in molecular biology are enabling us to acquire insights into the molecular level processes involved in preserving the cell membrane integrity[188].

Considering how important it is to understand the impact of cryopreservation on bioenergetic pathways and preservation of cell membrane integrity in HSPCs, I set out to investigate the impact of cryopreservation and different CPAs on biochemical pathways in CB HSPCs. This was achieved by using seahorse technology, and cell membrane integrity analysis using assays such as LDH and LP, to investigate the effect on viability and potency of HSPCs post thaw. The work conducted in our study will allow advancement in the field of cryobiology and offer the establishment for better cell preservation for HSCs and other cell types [224,225].

3.3. Materials and Methods

3.3.1. Collection and processing of CB units and CD34+ enrichment

UCB units were collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of Canadian Blood Services ahead of the study. Mononuclear cells were extracted from UCB using Ficoll-Paque Plus (GE, Pittsburgh, PA) following manufacturer instructions. CD34+ cells were isolated from mononuclear cells using EasySep™ Human CD34 Positive Selection Kit II (StemCell Technologies) according to the manufacturer's guidelines. CD34+ enriched cells were cryopreserved until use. CD34+ cells were supplemented with a cryoprotectant solution containing 40% IMDM, 50% FBS and 10% DMSO**.**

Cryovials were placed in an alcohol-based device (Mr. Frosty, Thermo Fisher Scientific, Waltham, MA, USA) in −80 °C freezer for 24 h. After 24 h, the cryovials were transferred to a liquid nitrogen Dewar flask for storage until thawed for analysis.

3.3.2. Bioenergetic analysis using seahorse technology

Agilent Seahorse XF analyzer (Seahorse XFe96; Agilent Technologies, Santa Clara, CA) was used to measure real time OCR in mitochondrial respiration and ECAR

during glycolytic respiration in DMSO/CPP cryopreserved HSPCs. Considering that the seahorse assay requires an abundant number of cells, primary cord blood CD34+ enriched cells were cultured for 7 days in media containing stem cell agonists for expansion to achieve an optimal cell number. A total of 3 CBUs were used for this assay. CD34+ enriched cells, which were cryopreserved with DMSO/CPP, were thawed, and then cultured using SFEM media for 10 days.

Then, they were plated at a concentration of 200,000 cells per well on 96 well Matrigel-coated Seahorse plates. Initially, baseline cellular OCR was measured. Next Oligomycin, a complex V inhibitor, was added and the resulting OCR was to derive ATP-linked respiration and proton leak respiration. Next carbonyl cyanide-p-trifluoromethox-yphenyl-hydrazon (FCCP), a protonophore, was added to collapse the inner membrane gradient, allowing the ETC to function at its maximal rate, and maximal respiratory capacity was derived. Lastly, Antimycin A and Rotenone, inhibitors of complex III and I, were added to shut down ETC function, revealing the non-mitochondrial respiration.

To conduct a mitochondrial stress test, Cellular OCRs were measured using an extracellular flux analyzer (Seahorse XFe96; Agilent Technologies, Santa Clara, CA). Briefly, the cartridge sensors were incubated overnight at 37°C in a hydration/calibration solution (XF Calibrant; Agilent Technologies). On the day of the assay, growth medium was changed to Seahorse medium (DMEM powder, 1 g/L D-glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, pH 7.4) for 1-hour at 37°C in base medium (phenol red-free low glucose DMEM [Sigma-Aldrich] supplemented with 3.5 g/l of cell-culture grade D-glucose [Sigma-Aldrich]). OCRs were measured to assess resting respiration, then ATP production-dependent respiration, maximal respiration, and non-mitochondrial oxygen consumption, after sequential injections of oligomycin (an ATP synthase inhibitor) at 2 µM (final concentration), carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP) (an

ionophore acting as a proton uncoupler) at 2 µM, and rotenone and antimycin A (complex I and complex III inhibitors, respectively) at 1 µM. Spare respiratory capacity, a measure of the ability of cells to respond to increased energy demand, was calculated by subtracting resting respiration from maximal respiration. ATP production-dependent respiration was calculated by subtracting the lowest OCR after oligomycin injection from resting OCR.

Proton leak, represented by basal respiration (i.e., mitochondrial respiration that is not coupled to ATP production), was calculated by subtracting the OCR corresponding to non-mitochondrial oxygen consumption from the lowest OCR after oligomycin injection. Non-mitochondrial oxygen consumption was subtracted from the reported resting respiration, maximal respiration, spare respiratory capacity, and ATP production dependent respiration. The ratio of maximal OCR as compared to basal OCR is known as spare respiratory capacity (SRC). SRC is the extra capacity available in a cell to produce energy under conditions of increased work or stress and is important for long-term cellular survival and function. For each parameter, OCR measurements were performed three times at 6-minute intervals.

Furthermore, a glycolytic stress test was performed. The measure of protons produced indirectly via lactate released from cells can be used as an indicator of glycolysis and is provided by ECAR. ECARs were measured using an extracellular flux analyzer. Briefly, the cartridge sensors were hydrated overnight at 37°C, as per the manufacturer's instructions, in a commercial calibration solution (XF Calibrant; Agilent Technologies). Sixty minutes prior to the experiment, cultured cells were incubated at 37˚C at ambient CO_2 in a glycolysis stress assay media (DMEM without glucose and glutamine supplemented with 143 mM NaCl, 0.5% phenol Red and 2 mM glutamine; pH 7.4). ECAR was measured at baseline followed by sequential injections of glucose (10 mM), oligomycin (2 µM), and 2-deoxy-D-glucose (30 mM) to measure glycolysis, maximal

glycolytic capacity, and non-glycolytic acidification, respectively. Lastly, 27 µL per well of Monensin (an ionophore which affects the sodium/potassium ATPase in cells and drives up the ATP demand) was injected to measure maximal ECAR.

Then, the cells were washed twice with 100 µL of 1X phosphate-buffered saline (PBS), then placed in 40 µL of RIPA buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% 31 NP-40, 10mM EDTA) containing 0.1% protease inhibitor cocktail (PIC) (Sigma Aldrich #P8340, 1:1000). A bicinchoninic acid (BCA) (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific #23225, Rockford, IL, USA) assay was used to determine protein concentration in each well and Seahorse assay measurements were normalized to protein content (µg/mL).

3.3.3. Reverse Transcription (RT-qPCR)

RNA was extracted using a PicoPure RNA extraction kit (Catalogue #1703517; Applied Biosystems by Thermo Fisher Scientific, Massachusetts, USA.). One microgram of RNA was isolated from corresponding CD34+ enriched cells cryopreserved with different CPAs and converted into cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Applied Biosystems by Thermo Fisher Scientific, Massachusetts, USA) as per manufacturers protocol. The cDNA was then used to perform RT-qPCR using the SsoAdvanced universal SYBR green supermix (Bio-Rad, California, USA).

Table 1: Primer sequences.

Table lists forward and reverse primers used in gene expression analyses. GAPDH was the control.

Primer	Sequence
PFKP forward	5'- CATCGACAATGATTTCTGCGG -3'
PFKP reverse	5'- CCATCACCTCCAGAACGAAG -3'

ALDOA forward	5'- GGTGTCATCCTCTTCCATGAG -3'
ALDOA reverse	5'- GTAGTCTCGCCATTTGTCCC -3'
GAPDH forward	5'- GAA GGT GAA GGT CGG AGT C -3'
GAPDH reverse	5'- GAA GAT GGT GAT GGG ATT TC -3'
PDK1 forward	5'- GGCTGGTTTTGGTTATGGATTG -3'
PDK1 reverse	5'- CTGGGAGTCTTTCTATTGAGTCTG -3'
HK2 forward	5'-GGGACAATGGATGCCTAGATG-3'

The assays were done using the CFX96 touch real-time PCR detection system (BioRad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene in the experiments to normalize the mRNA levels.

3.3.4. LDH Assay

This assay was conducted using LDH-Cytox Assay kit (Catalogue #426401, BioLegend Inc, San Diego, CA USA). This study was was performed to observe the potential impact of cryopreservation on the HSPC's membrane leakiness and was conducted by exposing fresh CD34+ enriched cells to different CPAs, including DMSO and DMSO, CPP, CSL and CN assess the impact on cell membrane leakiness and cellular damage (Figure 12).

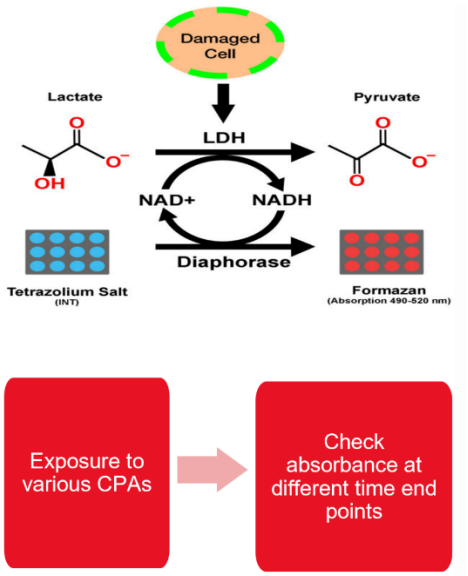


Figure 12: Experimental Design to assess the impact on cell membrane leakiness and cellular damage.

The LDH is a colorimetric assay that provides a reliable method for determining cellular cytotoxicity. Figure adapted from -https://www.cephamls.com/ldh-cytotoxicity-assay-kit-colorimetric-2/

For ideal results, I first performed a preliminary experiment to determine the optimal number of cells to ensure that the LDH signal was within the linear range. In the first step 100 µL of cell suspension CD34+ enriched cells were added (test done in triplicates) to each well of a flat-bottom 96-well tissue culture plate except the "background control" wells. Further, 100 µL of cell culture medium (IMDM with 2% FBS) was added to 'high control", 120 µL to "low control" wells, 20 µL to "test substance" wells, and 220 µL of medium in "background control" wells.

Then 100 µL different CPAs were added to cell suspension to study their impact on membrane integrity. Incubation of the plate was done in a 37°C CO_2 incubator for 30 minutes. Then, 20 µL of the Lysis Buffer was added to each well of the "high control'. Incubated the plate at 37°C for 30 minutes in a CO_2 incubator. Centrifugation was done at 250 × g for 2 minutes to precipitate the cells. Afterwards, 100 µL of the supernatant was transferred from each well to a new optically clear 96-well plate. The addition of 100 µL

of the working solution to all wells was done. Then, the samples were incubated at room temperature for 30 minutes in the dark. Further, added 50 µL of the Stop Solution to all wells to prevent any reaction beyond the time. The absorbance was measured at 490 nm using a spectrophotometer.

3.3.5. LP Assay

LP assay was done with the Lipid Peroxidation kit (C: Image-iT™ Lipid Peroxidation Kit) following manufacturer instructions. The Image-iT™ Lipid Peroxidation assay enables the detection of lipid peroxidation in live cells through oxidation of BODIPY™ 581/591 C11 reagent. The LP assay comprises an Image IT-Lipid peroxidation sensor, which was added to detect the effect of different CPAs on cellular peroxidation levels. This assay included cumene hydroperoxide as a positive control compound to induce lipid peroxidation in cells (Figure 13).

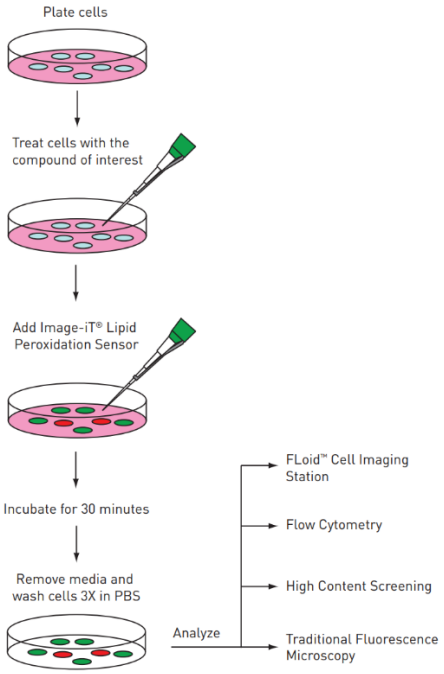


Figure 13: Workflow of LP assay.

The Image-iT™ Lipid Peroxidation assay enables the detection of lipid peroxidation in live cells through oxidation of BODIPY™ 581/591 C11 reagent. An Image IT-Lipid peroxidation sensor, which was added to detect the effect of different CPAs on cellular

oxidative stress levels. Figure adapted from [Lipid Peroxidation Image-iT™ Lipid Peroxidation Kit, for live cell analysis # C10445I]

Leukemic KG1 and CD34+ enriched cells were centrifuged at 300g for 8 minutes and resuspended in 100 µL SFEM media. Incubation of cells with different CPAs- DMSO, CPP-STEM, CSL, CN was done via incubation for 30 minutes. The next step was the addition of cumene hydroperoxide (component B) to the cells at a final concentration of 100 µM and incubated at 37°C for 2 hours to prepare the positive control sample. Then, the image-iT® lipid peroxidation sensor (component A) was added at a final concentration of 10 µM to the cells, followed by incubation for another 30 minutes at 37°C.

Afterwards, the cells were washed with PBS solution as described in chapter 2. For analysis, the reading was performed at an excitation/emission of 581/591 nm (Texas Red® filter set) for the reduced dye and at an excitation/emission of 488/510 nm (traditional FITC filter set) for the oxidized dye. The ratio of the emission fluorescence intensities at 590 nm to 510 nm gives the read-out for lipid peroxidation in cells. For accurate determination of lipid peroxidation quantification, the ratio of intensity in Texas Red® channel to the intensity in FITC channel using flow cytometry was calculated.

3.4. Results

3.4.1. Impact of cryopreservation on bioenergetics of HSPCs

Firstly, a pilot test to study the impact of CPA on metabolism of CD34+ enriched cells using the Seahorse technology was conducted to identify the optimum cell concentration to be used. This assay revealed that a dose of 200,000 CD34+ cells was required per well to get results within range (data not shown). Subsequent tests were done comparing HSPCs cryopreserved with DMSO vs CPP. Before each assay, the CD34+ cells were thawed and expanded for a week in a cell culture medium that support stem cell and

progenitor expansion[226]. Respiratory parameters- OCR and ECAR were then measured in real time on day 7 (Figure 14).

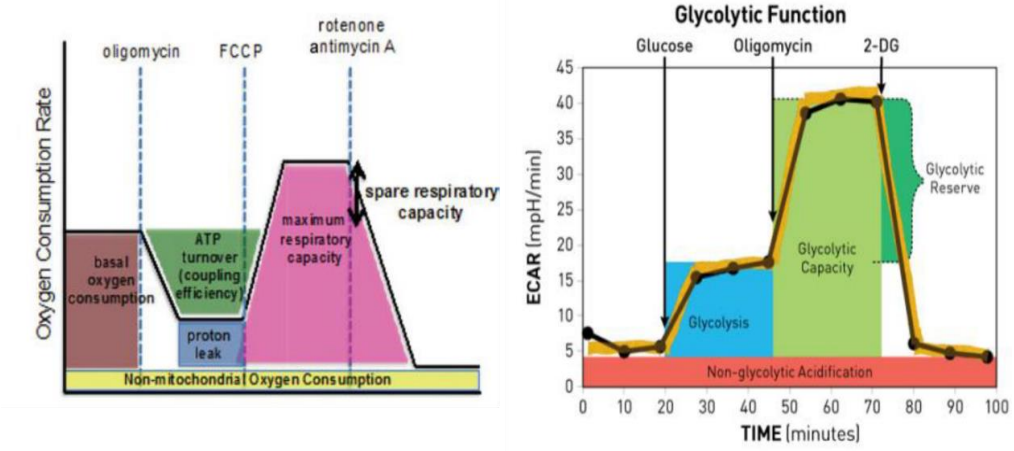


Figure 14: Overview of bioenergetic profile measurement with Seahorse Assay.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), which are indicators of glycolysis and mitochondrial respiration, respectively, are both simultaneously measured by the Seahorse XF analyzer. Figure adapted from Agilent.com seahorse Protocols [60].

The mitochondrial stress results revealed a significant increase in Spare respiratory capacity within cells frozen with DMSO in comparison with CPP. (Figure 15A). Overall, the data revealed that when HSPCs frozen with DMSO or CPP were subjected to respiratory monitoring, spare respiratory capacity was superior in cultured cells derived from CD34+ cells cryopreserved with DMSO (Figure 15A). Next, glycolytic test was performed using expanded HSPCs cryopreserved with DMSO and CPP. The glycolysis stress test medium contained no glucose or pyruvate and the ECAR was measured (Figure 15B). The cells cryopreserved with DMSO revealed a significant increase in ECAR after the 4th injection. The assay demonstrated a significant rise in maximal glycolytic capacity for DMSO (Figure 15B). Together, the data obtained by mitochondrial and glycolytic stress tests indicated that DMSO cryopreserved HSPCs had the highest OCAR and ECAR.

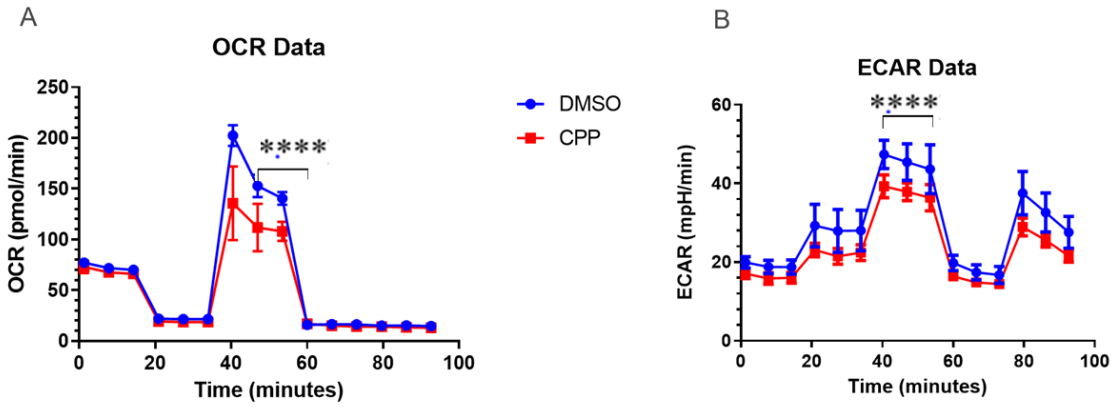


Figure 15: Assessing the impact of DMSO and CPP on mitochondrial bioenergetics and glycolysis in HSPCs.

DMSO/CPP cryopreserved HSPCs were cultured for 7 days and then measurement of OCR and ECAR in real time was performed using Seahorse apparatus. A) The Graph of the mitochondrial stress test displaying the four key parameters of mitochondrial function (basal respiration, ATP turnover, proton leak, and maximal respiration) with 4 sequential injection of drugs- Oligomycin, FCCP, Rotenone/Antimycin A and Monensin. B) Graph of the glycolytic stress test detailing the parameters of cellular glycolysis obtained with the sequential use of above-mentioned drugs. Data represents the mean +/- SEM, n=3. Two-way ANOVA with Greenhouse–Geisser correction and Tukey's multiple comparisons test. ****P < 0.0001.

Next, I investigated the impact of CPAs on the expression of genes that mediate glycolysis by RT-qPCR assay. Freshly cultured HSPCs were used as positive control. In line with the findings from the seahorse test, the RT-qPCR data indicated a relatively higher expression of glycolytic genes in DMSO cryopreserved HSPCs as compared to control HSPCs and HSPCs frozen with CPP (control) (Figure 16). There was also a noted reduction of glycolytic gene expression in CSL HSPCs. Interestingly, CSL had a glycolytic gene expression which was similar to fresh cells acting as control in the experiment (Figure 16).

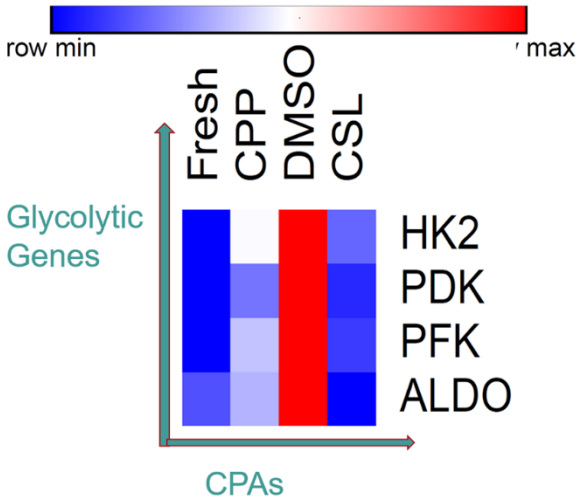


Figure 16: Impact of CPAs on glycolytic genes in cryopreserved HSPCs.

HSPCs were cryopreserved with different CPAs and RT-qPCR was performed post thaw. Mean of 3 triplicates for each sample. n=3

3.4.2. Influence of CPAs on hematopoietic cell membrane integrity

The LDH and LP experiments were conducted to evaluate impact of the different CPAs tested in Chapter 2 on the membrane integrity of HSPCs to identify a potential explanation why some work better than other with those cells. The optimization of protocols and preliminary experimentation was done using KG1 cell line. The data obtained in KG1 experiments indicated that DMSO emerged to be the CPA with lowest LDH leakiness levels suggesting its least harmful impact on cell membrane of HSPCs LDH levels were highest in DMSO samples followed by CPP and were significantly lower for CN when compared with DMSO control (Figure 17A). The LDH results using HSPCs

showed that the different CPAs had no significant impact on cellular membrane integrity pre freeze as LDH levels were similar to that seen in untreated cells (Figure 17B).

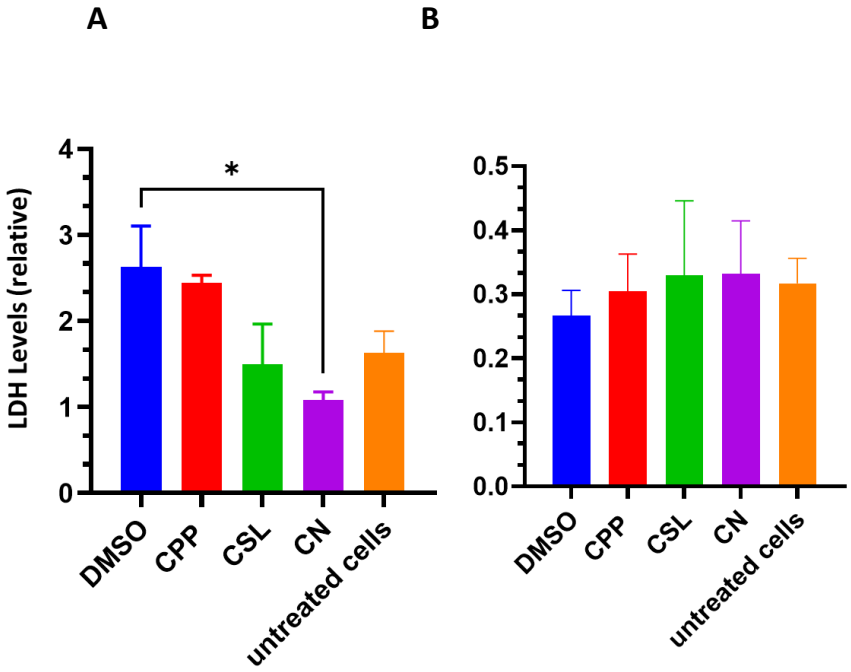


Figure 17: Impact of CPAs on cell membrane leakiness of HSPCs before cryopreservation.

LDH levels in A) KG1 cell line supernatant *<0.05 and B) CB CD34+purified cell supernatant. Cells were exposed to varying CPAs and leaked LDH was measured. Mean ± SEM, n=3. * Signifies p<0.0005 vs other conditions (Anova).

LP assay was used to measure the oxidative degradation preceding cell damage in HSPCs. The data from the LP assay revealed that KG1 cells exposed to various CPAs had rather comparable peroxidation levels (Figure 18A). In Figure 18 B, the results followed a corresponding trend amongst all CPAs having similar peroxidation levels. Though, slightly lower stress levels were detected when CB HSPCs were exposed to CPP and CN. The LP assay results indicated that there were no significant differences observed in the peroxidation levels of HSPCs exposed to CPAs in comparison to control sample, which was not exposed to any CPA.

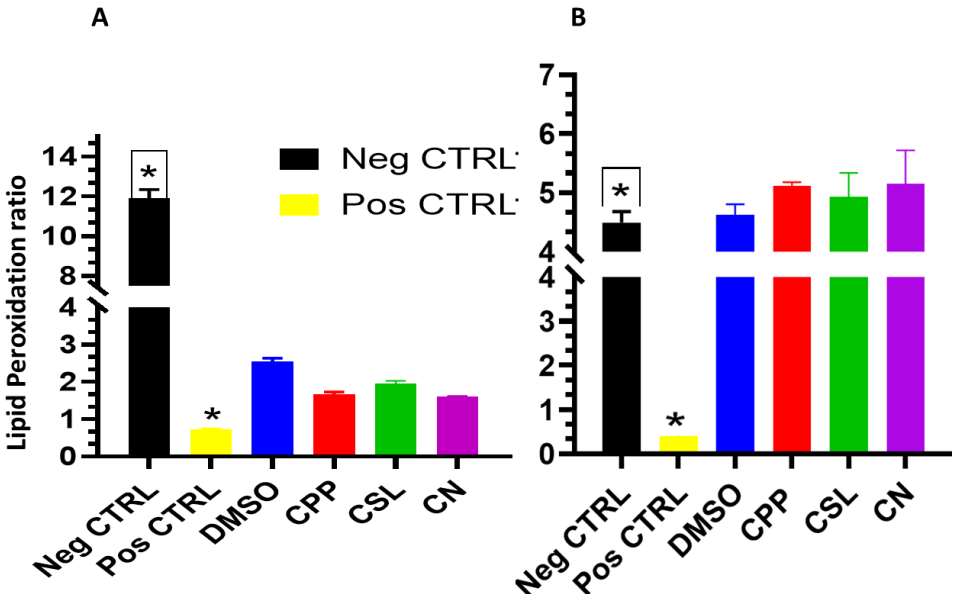


Figure 18: Impact on cellular peroxidation levels.

HSPCs were exposed to varying CPAs and oxidation/reduction ratio was determined. Negative control comprises of cells without any CPA while positive control had cumene hydroperoxide to activate lipid peroxidation. A) Lipid Peroxidation Ratio in CD45+CD34+ cells in KG1 cell line. B) Lipid Peroxidation Ratio in CD34+ enriched cells. SEM, n=3. * Signifies $p<0.0005$ vs other conditions (Anova).

3.5. Discussion

Cryopreservation can induce various functional consequences in cells; thus, it is important to comprehend how these effects influence the subsequent function of cells post thaw[227]. Furthermore, CPAs are indispensable to limit irreversible damages to cells during the freezing and thawing periods that further impacts cell viability[191]. In my chapter 2, I investigated the impact of different CPAs on viability and potency of CB HSPCs post thaw. In this chapter 3, I wanted to study the impact of these CPAs on the key biochemical mechanisms important for the survival of CB HSPCs. Therefore, I tested the hypothesis that CPAs can directly impact bioenergetic pathways involved in regulation of HSPCs in CB.

For this, I tested for the first time to my knowledge the impact of two different CPAs (DMSO and CPP) on the main bioenergetic pathways, namely mitochondrial

respiration, and glycolysis in primary CB HSPCs. I selected these two CPAs since I had identified CPP as valuable alternative to DMSO. This new DMSO-free CPA provided similar protection to HSPC than DMSO based on the results obtained in chapter 2 for cell viability and potency post thaw[226]. Investigations using the Seahorse technology indicated that the mitochondrial respiratory capacity of CD34+ cells cryopreserved with DMSO was significantly superior to CPP. Moreover, it also revealed that the glycolytic capacity of DMSO-samples was significantly superior to that of CPP-samples.

My results suggest that depending on the type and concentration of CPAs, the temperature and duration of exposure, and the cell type, a CPA can either impair or enhance the metabolic activity and function of HSPCs by altering their substrate utilization, energy production, and redox balance. Despite these differences, CPP showed similar capacity to preserve CD34+ cells post thaw and CFU (Chapter 2). This is consistent with CPP-HSPCs having normal bioenergetic responses in the seahorse assay. Indeed, CPP-HSPC responded normally to the different inhibitors as the slopes seen for both DMSO-HSPCs and CPP-HSPCs were parallel to each other and only differed in the magnitude of some responses. Functionally, this could translate into reduced capacity in generation of energy in CPP-HSPCs. Whether these effects govern or limit in part or not the efficacy of these 2 CPAs to cryoprotect CB HSPCs is unclear at this time and will need to be investigated in future work.

It is also possible that cryopreservation may impact either one of those bioenergetic process, but this will also need to be investigated in a subsequent study. Indeed, several studies have investigated the impact of cryopreservation on cell energetic metabolism. A study by Kocaoemer *et al*., that used similar technology to measure the OCR and ECAR of human adipose-derived stem cells indicated that cryopreservation reduced the basal and maximal respiration rates but increased their glycolytic rate and reserve[228]. Another study

by Keane *et al.,* showed that cryopreservation decreased mitochondrial respiratory rates of MSCs. This indicates that cryopreservation may impair the bioenergetic efficiency and adaptability of stem cells post thaw[152].

Next, I measured the expression levels of glycolytic genes by RT-qPCR to study the impact of cryopreservation and CPAs on glycolysis in HSPCs. I tested 3 CPAs here-CPP, DMSO and CSL. Interestingly, expression of glycolytic genes was at the highest in DMSO-protected HSPCs which corroborated the trend observed in Seahorse tests. Consistent with the literature[166,182], the level of glycolytic genes in fresh HSPCs were very low. Noteworthily, CSL and to a lesser extent, CPP, had the lowest level of glycolytic genes of all cryopreserved samples suggesting perhaps that glycolysis might not play a prominent role in energy production when HSPCs are cryopreserved using CSL as CPA. To date, not many studies have investigated the impact of different CPAs on bioenergetic pathways at both cellular and molecular level. Notably, my study is one of the first of its kind to investigate the effects of different CPAs on glycolytic gene expressions of cryopreserved CB HSPCs.

Loss in membrane integrity leads to necrosis, which is the main mechanism in loss of viability during the freezing and thawing cycle[162]. In Chapter 2, I showed that some CPA failed to protect CB HSPC from cryo-induced necrosis, with CN and PIM showing pronounce increase in necrotic CB cells post thaw[226]. Consequently, in this objective, the impact on cellular membrane integrity independently of freezing and thawing in HSPCs was investigated using two complementary assays. Results from the LDH assay revealed that there were no significant differences on cellular membrane leakiness between HSPC samples processed using different CPAs. The lack of differences indicated that the differences in CD34+ potency cryopreserved with the same CPAs observed in Chapter 2 were not due to a short-term physical damage and toxicity inflicted by the CPAs on the

cells before cryopreservation. Additionally, the LP assay was conducted to observe the impact of the CPAs on the peroxidation levels of HSPC's membrane. ROS mediate lipid peroxidation and produce 4-hydroxynonenal and other related products, which play an important role in the process of cell death, including apoptosis and autophagy[229]. My results with CB cells indicated that the cell membrane oxidative stress levels in HSPCs exposed to the CPAs were similar to the untreated control samples. This result suggests that oxidative damage of lipid residues in the HSPC' cell membrane is not a major issue to cell viability with the different CPAs tested. Together, these results reinforce the conclusion that CN and PIM to a lesser extent are poor CPAs for HSPC since they most likely fail to properly manage water molecule during freezing and/or prevent ice recrystallization during the freezing and thawing of CB HSPCs.

There are several limitations to the present work that I acknowledge; first, the low number of units tested due to time constraint and a shortage of CBU at the time of my work. It would be beneficial to increase the number of CBUs tested to acquire more robust results. The second limitation is that HSPCs were expanded for 7 days before the Seahorse assay was carried out. My data should therefore be confirmed with freshly thawed HSPCs whenever possible. Another limitation is the lack of a non-cryopreserved control sample to characterize the impact of cryopreservation on the two bioenergetic pathways which could be investigated once CBU become more readily available. Moreover, I did not explore potential changes at the protein level of key proteins implicated in glycolysis or mitochondrial respiration due to time constraints. Finally, the results obtained on cell membrane integrity and health could be confirmed with other assays, such as cytotoxicity (bioluminescent) and fluorescent microscopy[230].

In conclusion, the seahorse analysis that I conducted revealed that HSPCs cryopreserved with DMSO showed elevated mitochondrial and glycolytic function in

culture when compared to CPP-HSPCs counterpart. Conversely, there was no short-term impact on cell membrane integrity and peroxidation levels of HPSCs upon exposure to different CPAs. Studying the impact of cryopreservation on CB HSPC's biochemical activity such as done herein is necessary to bring up new knowledge regarding the role of factors such as CPAs in the bioenergetics of HSCs.

3.6. Acknowledgements

We wish to thank the staff at Canadian Blood Services Cord Blood for Research Program. This work was conducted in collaboration with Dr. Mary Ellen harper's laboratory, department of Biochemistry at University of Ottawa. Claire Fong McMaster, a master's student at Dr. harper's lab helped in doing seahorse experimentation work. R.K. was a recipient of Canadian Blood Services Doctoral Graduate Fellowship.

CHAPTER 4

EARLY ACTIVATION OF AUTOPHAGY IS INDISPENSABLE FOR THE SURVIVAL OF HSPCs DURING CRYOPRESERVATION

4.1. Summary

Background and aims: Autophagy is a very important biochemical mechanism and activated by cells in response to stress. Recent studies have indicated that changes in autophagy pathways can impact cells during cryopreservation. However, this topic remains uninvestigated in HSPCs at the present time. In addition, the impact that CPAs may have on autophagy in cells has not been addressed yet. Hence, I sought to address the impact of cryopreservation and CPAs on autophagy in HPSCs and investigate the functional importance of autophagy in HSPC survival and potency post thaw.

Methods: Autophagy flux, viability and potency analysis were tracked by cytometry in fresh CB HSPC and following cryopreservation and thawing. Autophagy activators and inhibitors were used to modulate autophagy pre-freeze. The impact of different CPAs (DMSO, CPP and CSL) on autophagy in HSPCs was also investigated.

Results: Autophagy flux levels were significantly increased following the cryopreserved and thawing of HSPCs. Moreover, viability analysis and potency assays of CB HSPCs revealed that inhibition of autophagy with 3-MA during cryopreservation leads to low recovery and low potency of HSPCs post thaw. Furthermore, the autophagy flux data suggests that the tested CPAs may have a different impact on autophagy in CB HSPCs.

Conclusions: The results provide compelling evidence that early activation of autophagy is critical for preserving the functional capacity of CB HSPCs post thaw.

4.2. Introduction

Autophagy is a highly conserved cellular process by which cytoplasmic components are sequestered in autophagosomes and delivered to lysosomes for degradation. Recent literature has been confirmed that autophagy plays a crucial role in the survival and function of HSCs[26,] T-cell differentiation, and responsiveness to cytokine stimulation[179](Figure 19). Moreover, dysfunctions in autophagy have been associated with a variety of pathologies including cancer[177,178].

However, the role played by autophagy during cryopreservation of HSPC is presently unclear, and it is also unknown whether CPA solutions will impact such an important biochemical pathway normally used by cells to sustain survival following stress. My previous work showed that some CPAs work well with HSPCs while others failed completely (Chapter 2), and that surprisingly some of those CPAs can modulate the energetic pathways used by cells post thaw (Chapter 3). In this Chapter, I will test my hypothesis that autophagy is important for the post thaw survival of HSPCs, and that CPA can influence the capacity of cells to undergo autophagy.

Interestingly, under clinical stress, particularly in HSCT and mobilization after chemotherapy, HSCs can rapidly produce new mature blood cells. Therefore, HSC requires more autophagy activity than other cells to preserve their unique features and to survive long-term survival[231] .As a result of the growing use of autophagy-modulating agents in the clinic, it is imperative to better understand the functions of autophagy in HSCs[232].

A study by Moretensen *et al.,* demonstrated that *ATG7* deletion in the hematopoietic system resulted in the loss of normal HSC functions, a severe myeloproliferation, and death of the mice within weeks[185]. ATG7-deficient cells failed to reconstitute the hematopoietic system of lethally irradiated mice. Consistent with loss of

HSC functions, the production of both lymphoid and myeloid progenitors was also impaired[185]. Similarly, deletion of Atg12 in HSCs increased mitochondrial content, which was accompanied by an activated metabolic state and enhanced myeloid differentiation. Moreover, Atg12-deficiency impaired self-renewal potential of HSCs[36]. Furthermore, within the murine system, HSCs induce autophagy after in vivo calorie restriction and ex vivo cytokine withdrawal, driven by FOXO3, a transcription regulator[198]. A study by Rozman *et al.,* demonstrated that expression levels of BECN1 and LC3-II genes were highly upregulated in murine lupus B and T cells[77].

Thus, autophagy is important for maintaining cellular homeostasis of HSCs [233]. But the impact of cryopreservation on autophagy in HSCs is not well understood[180]. However, autophagy can be a protective mechanism for stem cells from cellular stress due to the reduced potential of stem cell regeneration and increased degenerative diseases in aging. Accordingly, a recent review article stated that "autophagy is a key mechanism for cellular survival and function, and its modulation may be a novel strategy to improve the quality of cryopreserved HSCs"[202].

Additionally, recent work by our group and others[234] showed that autophagy plays a significant role in the maintenance of HSCs during storage and aging. Specifically, revealing that rescuing autophagy within CD34+ cells during prolonged storage of CBU at room temperature rescues the viability and function of HSPCs (Jahan *et al.,* manuscript in preparation).

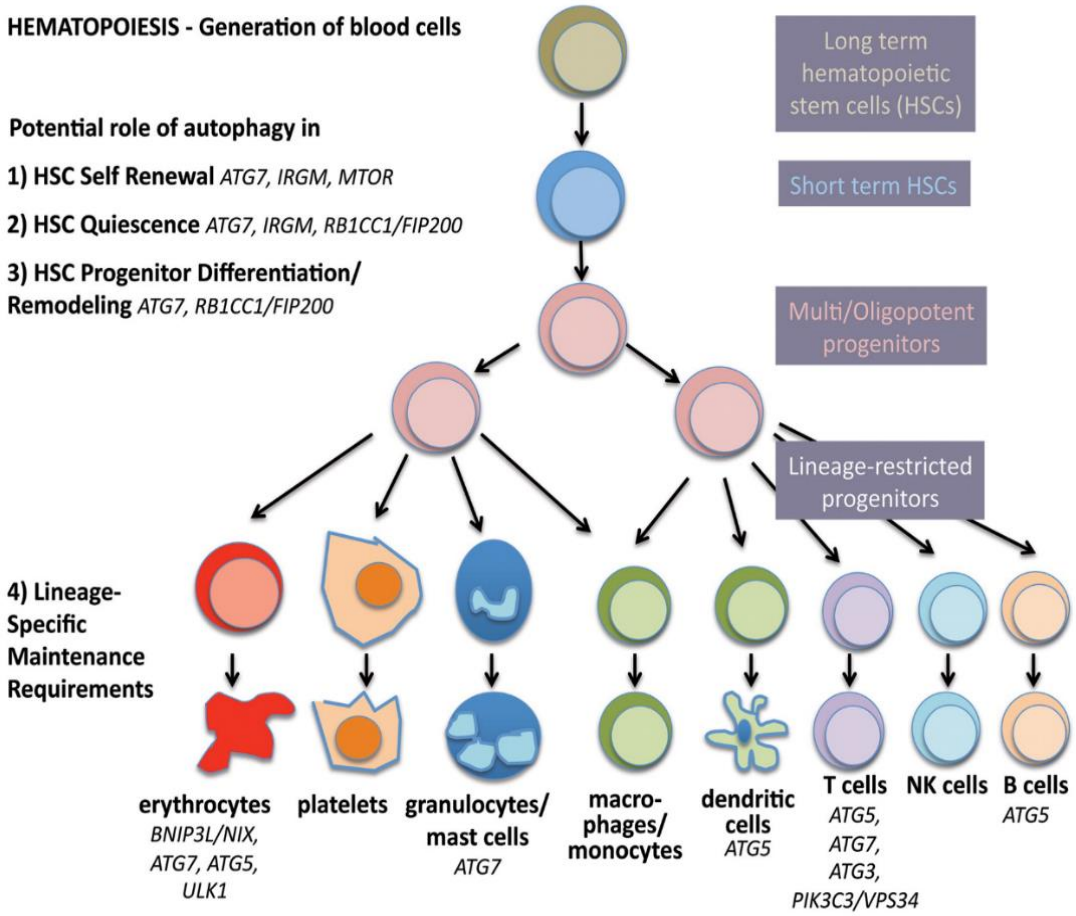


Figure 19: Autophagy in stemness of HSCs.

The two characteristics of stemness, self-renewal and quiescence in HSCs, are influenced by autophagy. Figure adapted by Guan *et. al.*, 2013[178]

In my work, I have selected a panel of autophagy modulators (rapamycin, trehalose, bafilomycin A1 and 3-MA) as tools to investigate the modulatory activity of autophagy on cryopreserved CB HSPCs. Rapamycin, a well-known inducer of autophagy and a lipophilic macrolide antibiotic, can suppress the activity of mTOR[235]. Decreasing the signaling and activity of the mTOR pathway increases the expression of autophagy genes leading to an increased activity[173]. Moreover, it was recently revealed that autophagy was downregulated in cryopreserved MSCs and that restoring autophagy with rapamycin could enhance their immunomodulatory function[232]. Trehalose is another autophagy activator and has the capacity to protect labile proteins and the cellular membrane from damage and denaturation

due to oxidative stress[236,237]. It is also a well-known extracellular CPA. Indeed, a series of studies showed that trehalose improves cell survival compared to the standard freezing protocol and can preserve the structural integrity of cells during the freezing and thawing process [130,238]. The mechanism by which trehalose protects the lipid membrane and the cell from oxidative stress is currently unknown.

Bafilomycin A1, is a late-stage autophagy inhibitor that blocks the fusion of autophagosomes and lysosomes, which prevents the degradation and recycling of cellular components. Yuan *et al.* also revealed that bafilomycin A1 blocks autophagic flux leading to the accumulation of p62 and LC3-II in acute lymphoblastic leukemia cells[239]. Finally, 3-MA is a phosphatidylinositol 3-kinase (PI3K) inhibitor[190] widely used as an autophagy inhibitor. PI3K plays a critical role in several biological processes which include the regulation of mTOR activation, a regulator of autophagy[240]. The mechanism of 3-MA involves blocking the formation of autophagosomes by inhibiting the class III PI3K complex[190].

In summary, recent studies have demonstrated the importance of autophagy in normal HSC biology, and for the survival of HSC during stress. However, more research is needed to elucidate the impact of cryopreservation and of CPAs on autophagy in HSPCs. Hence, I sought to answer these two questions and to investigate the role of autophagy on the survival and potency of HSPCs post thaw.

4.3. Methods

4.3.1. CBU Processing and CD34+ enrichment

UCB was collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of Canadian Blood Services ahead of the study. Mononuclear cells were extracted from UCB

units using Ficoll-Paque Plus (GE, Pittsburgh, PA) following the manufacturer's instructions. CD34+ cells were isolated from mononuclear cells using an EasySep™ Human CD34 Positive Selection Kit II (StemCell Technologies) according to manufacturer's guidelines. Then, CD34+ enriched cells were supplemented with a cryoprotectant solution containing 40% IMDM, 50% FBS and 10% DMSO and cryopreserved in an alcohol-based device (Mr. Frosty, Thermo Fisher Scientific, Waltham, MA, USA), which was stored in a −80 °C freezer for 24 h. After 24 h, the cryovials were transferred to a liquid nitrogen Dewar flask for storage until thawed for analysis.

4.3.2. Autophagy Activators/Inhibitors

Rapamycin (500 nM) and Trehalose (Final concentration-100µM) were obtained from Cayman Chemical Co. Bafilomycin A1 (Final concentration-1 µM) and 3-MA (Final concentration-10nM) was obtained from Millipore (Sigma). The modulators were used in leukemic KG1 cells and CB HSPCs to observe the impact of autophagy modulation during cryopreservation. The drug dosage was optimised in dose-response experiments in KG1 cells to identify the optimal concentration to either activate or block autophagy.

4.3.3. Autophagy modulators treatment of Kg1 cells and CB cells

All CBUs (n=3) were obtained from the Canadian Blood Services CB for Research Program after obtaining institutional ethical approval (REB PROTOCOL REFERENCE # 2013.016 – Hematopoietic stem cells and transfusion medicine) and written informed consent from CB donors. Each CBU had a minimum volume of 50 mL and a TNC count greater than 0.9 $X10^9$ but inferior to 1.5 $X10^9$. CB buffy coat cells (i.e., CB cells) were processed as discussed in Chapter 2 and 3- processing of CBU units. Autophagy modulators were added to the respective cells and autophagy flux measured after 5.5 hours of treatment (pre-freeze), or cells were frozen with DMSO, and modulators and autophagy flux measured post thaw as indicated.

4.3.4. Impact of different CPAs on autophagy in CD34+ cultured cells

CD34+ enriched cells were expanded in 24-well plates (Corning, New York, United States) in STFL media comprised of serum-free expansion medium (SFEM) (StemCell Technologies, Vancouver, Canada) supplemented contained 100ng/ml of SCF, TPO, and FLT-3 respectively, 10µg/ml of low-density lipoprotein (LDL, StemCell Technologies, Vancouver, Canada), and 1% Penicillin- Streptomycin (Gibco, CA, USA). The cytokines SCF, TPO and FLT-3 were obtained from Peprotech (Rocky Hill, NJ, USA). The following stem cell agonists were added to CB cultures to favor HSPC retention and expansions; StemRegenin1 (SR1) at 750 nM, UM171 at 35 nM (StemCell technologies). Cell cultures were kept at 37°C in a humidified 5% carbon dioxide (CO_2) incubator. After 10 days of culture, CB cells were collected and divided into 9 conditions (3 with only CPAs, 3 with CPAs and Trehalose, 3 with CPAs and 3-MA) to investigate the viability and autophagy levels pre-freeze and post thaw.

First the cells were treated with autophagy modulators for 5.5 hours and then exposed to CPAs for 30 minutes before freezing. DMSO, CryoScarless (CSL, BioVerde) or CPP (Ad Infinitum) were used to protect CD34 + enriched cells with the addition of 100 uM trehalose or 10 mM 3-MA to study autophagy modulation in CD34+ enriched cells. Twenty percent of total volume of DMSO or CPP was added respectively. For CSL, cells were centrifuged at 500xg for 5 mins., then the cell pellets were resuspended in CSL. Cells were then frozen until use as indicated.

4.3.5. Cryopreservation and Thawing

Samples were frozen down in Mr. Frosty Freezing container (Thermo Fisher Scientific, Massachusetts, USA) overnight at -80°C then transferred into liquid nitrogen. KG1 and CD34+ frozen samples were placed in a water bath at 37°C to thaw for 30 seconds. A 1:1 dilution with Plasmalyte A (Baxter Corporation, DIN 02339358) + 4%

Albumin (Human Albumin 25% solution, DIN 02274663) was added and allowed to equilibrate for 30 minutes at RT.

4.3.6. Autophagy Flux Assay

Autophagy Flux Analysis was performed following manufacturer's instructions given in CYTO-ID® Autophagy Detection Kit (. ENZ-51031, Enzo Life Sciences). The autophagy flux assay was first optimized using KG1 cells. Once the assay was optimized, autophagy flux levels were measured within fresh and freeze/thawed KG1 cells, TNCs and subsequently CD34+ cells treated or not with autophagy modulators as indicated. Expanded CD34+ cells were treated with CPAs (DMSO control, CPP, CSL) and autophagy modulators (Trehalose, 3-MA) for 30 minutes for pre freeze analysis. For post thaw analyses, the cells were thawed immediately, and flux levels measured. Then, the cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The pelleted cells were resuspended in 1X Assay buffer. Also, each cell sample was resuspended in 250 µL of 1X Assay Buffer. 250 µL of the diluted CYTO-ID® Green stain solution was added to each sample and mixed well. Stained cells were then incubated for 30 minutes at RT or 37°C in the dark. After treatment, the cells were collected by centrifugation and washed with 1X Assay Buffer and cell pellets were resuspended in 500 µL of fresh 1X Assay Buffer. The cells were analyzed in the green (FL1) or orange (FL2) channel of a flow cytometer.

4.3.7. Flow cytometry analysis

Flow cytometry was carried out using a FACS-Attune device (Thermo Fisher)[215]. Dead cells and debris were gated out by the forward and side-scatter and/or Sytox-AAD staining (Life Technologies). Compensation was carried out with fluorescent labelled microbeads (Molecular Probes- Thermo Fisher Scientific Inc.) and fluorescent intensity minus one stained sample (FMO) were used as controls to set gates. An analysis of the $CD34^+$ cell percentage of each sample was performed using two independent platforms.

PocH-100i hematology analyzer was first used to obtain the cell counts. Later, TNCs/ CD 34+ enriched cells were stained with CD34, CD45 and 7-AAD antibodies and analyzed using flow cytometry to calculate the frequency of CD34+ and CD45+ cells as defined by the ISHAGE gating strategy[215].

Unless specified otherwise, CD34, CD45 and 7-AAD antibodies were purchased from Becton Dickinson Pharmingen (Mississauga, Ontario, Canada). Antibodies were added to CB samples containing approx. 1.5-2.0 X 10^6 cell in 100 µL and incubated for 30 min at 4°C in the dark. CB cells were then lysed by adding 1 mL of lysis buffer (BD Biosciences) followed by an additional incubation for 10 min in the dark at room temperature Next, cells were pelleted (250 X g /6 min) and suspended in in IMDM with 2% FBS solution or in annexin V binding buffer (for viability assay) followed by staining with annexin V Alexa 488 and a dead cell stain kit (SYTOX AADvanced, Thermo Fisher Scientific) following the manufacturer's instructions.

4.3.8. CFU assay

CFU assays were performed by plating CB TNCs (50 000 per plate) in methylcellulose-based media (MethoCult H4434; STEMCELL Technologies, Vancouver, Canada) in duplicate and incubating for 2 weeks in a humidified atmosphere (5% carbon dioxide) at 37°C. Colonies were scored by microscope based on standard morphological criteria: CFU total, BFU-E, CFU-GM and CFU-GEMM progenitors.

4.3.9. The IL3-pSTAT5 assay

Aliquots of thawed CB samples were prepared as follows: 25 µL of CB were diluted with 85 µL of Iscove modified Dubecco's medium supplemented with 10% fetal bovine serum (GIBCO) in a 96-well plate. For each sample, one well was used as an unstimulated control, and the second well was stimulated by adding 1 µL of 10 µg/mL IL-3 (Stem Cells Technologies) at a final concentration of 100 ng/mL. Plates were incubated

for 20 minutes at 37°°C, 5% CO_2. Samples were then transferred to tubes containing 20 volumes of prewarmed buffer (BD Bioscience) and incubated for 10 minutes in a 37 °C water bath. Following centrifugation at 500× g for 8 minutes, pelleted cells were resuspended in 90% methanol–10% PBS then incubated at room temperature for 20 minutes. After centrifugation at 500× g for 8 minutes, the cell pellets were washed with PBS–1% BSA. The cell pellets were suspended in 50 µL of PBS–1% BSA, mixed with 10 µL of CD45-FITC/CD34-PE mix (clone 2D1 and 8G12, BD Biosciences) and 10 µL of anti-STAT5 (pY694) (Alexa Fluor 647 [clone 47/Stat5(pY694], BD Biosciences). Staining was done overnight at 4˚ °C. The next day tubes were centrifuged at 800× g for 8 minutes and cell pellets suspended in PBS–1% BSA and analyzed using flow cytometry.

The CD34 population was identified based on ISHAGE guidelines[96]. Level of STAT5 phosphorylation was then measured for CD45+CD34+ population. The unstimulated control was used as a negative control to establish the baseline endogenous pSTAT5 activation. These limits were established for each sample to have 1% or less positive cells in the unstimulated sample. Cells in the stimulated sample showing a STAT5 phosphorylation level above this basal level threshold were deemed responsive to IL-3 reactivity, therefore permitting the measurement of the proportion of IL-3–responsive CD34 cells in the sample[91,93,96,221].

4.3.10. RT-qPCR

RNA was extracted using a PicoPure RNA extraction kit (Catalogue #1703517; Applied Biosystems by Thermo Fisher Scientific, Massachusetts, USA.). One microgram of RNA was isolated from corresponding CD34+ enriched cells cryopreserved with different CPAs and converted into cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Applied Biosystems by Thermo Fisher Scientific, Massachusetts, USA) as per manufacturers protocol. The cDNA was then used to perform RT-qPCR using

the SsoAdvanced universal SYBR green supermix (Bio-Rad, California, USA). The assays were done using the CFX96 touch real-time PCR detection system (BioRad). GAPDH was used as the housekeeping gene in the experiments to normalize the mRNA levels.

Table 2: Primer sequences.

Table lists forward and reverse primers used in gene expression analyses of autophagy genes. GAPDH was the control.

Primer	Sequence
ATG4 forward	5'- AGATTGGAGGTGGACACAAAG-3'
ATG4 reverse	5'-ACGTATCGAAGACAGCAAGC -3'
ATG12 forward	5'- AGAGCGAACACGAACCATC-3'
ATG12 reverse	5'- CCATCACTGCCAAAACACTC -3'
GAPDH forward	5'- GAA GGT GAA GGT CGG AGT C -3'
GAPDH reverse	5'- GAA GAT GGT GAT GGG ATT TC -3'
BECN1 forward	5'- CTGCCGTTATACTGTTCTGGG-3'
BECN1 reverse	5'- TCTTGCCTTTCTCCACATCC-3'
ULK2 forward	5'- CGGATGACTTTGTTTTGGTGC-3'
ULK2 reverse	5'- AGGAACTGGAATTGGTGCTG-3'
PINK1 forward	5'- TGAACACAATGAGCCAGGAG-3'
PINK1 reverse	5'-CAGCACATCAGGGTAGTCG-3
P62 forward	5'-CGGGTTTAATTTTGGAGGCAC-3'

P62 reverse	5'-TTGTGGCTGGTTGGAAGG-3'

4.3.11. Statistical analyses

Statistical analyses were performed using the statistical software Prism 8 (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were done using two tailed Students t-test. One-way or two-way ANOVA was used when comparing the mean between more than 2 groups when appropriate. $P< 0.05$ was considered significant. Data are presented as standard error mean (SEM) unless otherwise stated.

4.4. Results

4.4.1. Optimization of autophagy investigation

Firstly, I used KG1 cells to identify the optimum dose and length of treatment for each autophagy modulators to be used to study the importance of autophagy in the process of cryopreservation. This work also ensured that the autophagy modulators had the expected effects on autophagy. A cytometry assay (Cyto-ID dye autophagy flux assay) was used to measure the autophagy flux levels in cell samples.

The results obtained with fresh KG1 cells confirmed that autophagic flux levels were significantly increased by the autophagy activator rapamycin and trehalose when compared to untreated KG1 cells that served as baseline (Figure 20A). No blunting under basal condition in autophagy was seen with the autophagy inhibitors bafilomycin A1and 3-MA (Figure 20A). Cultured KG1 cells were also exposed to the CPAs DMSO and CPP; the data showed similar autophagy flux levels in KG1 cells treated with both the CPAs. However, autophagy flux was slightly superior in CPA treated KG1 cells [MFI of 384 and 452 for DMSO and CPP, respectively] when compared to untreated samples [MFI of 323] though the differences were not significant (Figure 20A).

Next, KG1 cells were cryopreserved with DMSO/CPP or after autophagy modulator treatments (DMSO only and frozen with modulators) to investigate the functional importance of autophagy during cryopreservation and post thaw cell recovery. Autophagic flux assay were done immediately after thawing (post thaw 0) and viability analyses after thaw and 24 hours after thawing (post thaw 24+) to detect late onset loss of cell viability (Figure 20B). Comparison of autophagy flux levels in fresh vs. post thaw KG1 samples demonstrated a clear induction of autophagy following thawing of frozen samples in DMSO samples (MFI of 257 and 1489 in fresh Vs post thaw, respectively, $p<0.0001$, $n=3$) and in CPP samples (Figure 20A-B).

Next, I investigated the impact of the modulation of autophagy on viability and recovery of KG1 cells post thaw. Surprisingly, despite the autophagy activators being present during the freezing and thawing process, no significant increase in autophagy flux above untreated control samples was detected (Figure 20B). Moreover, viability analysis showed that addition of the activators failed to improve the efficacy of the cryopreservation at least with DMSO as shown by similar post thaw cell viability (Figure 20C). On the other hand, bafilomycin A1 and 3-MA treated KG1 cells showed a significant reduction in autophagy flux post thaw (Figure 20B), which is consistent with the induction of autophagy during the thawing process as indicated above. However, both inhibitors had different impact on cell viability; the early autophagy inhibitor 3-MA significantly reduced cell viability while the late autophagy inhibitor bafilomycin A1 samples had normal viability and cell recovery (Figure 20C-D). Altogether, these assays confirm the functionality of the autophagy modulators and provided the 1st insights into the role of autophagy in cryopreservation of hematopoietic cells.

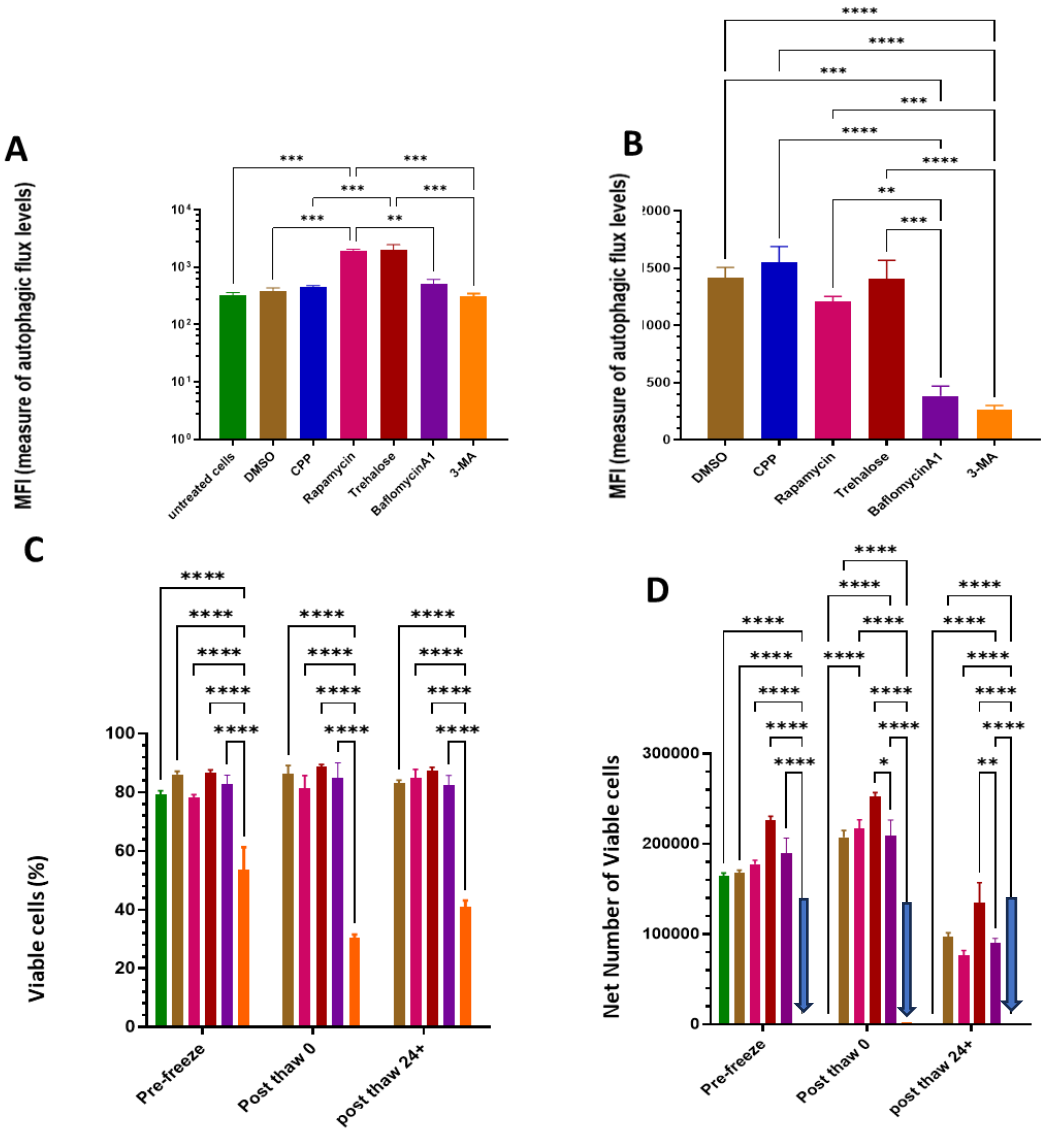


Figure 20: Impact of autophagy activators and inhibitors on autophagy.

(A) Autophagic flux detection using Cyto-ID dye in cultured KG1 cells. B) Autophagic flux detection using Cyto-ID in KG1 cells post thaw. C) Post thaw viability analysis of KG1 pretreated or not with autophagy modulators. D) Net number of viable KG1 cells post thaw. Data represent the mean +/- SEM. n=3 Two-way ANOVA with Greenhouse–Geisser correction and Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

4.4.2. Investigation the functional importance of autophagy on the cryopreservation of HSPCs

Next, I sought to characterize the role of autophagy in the cryopreservation CB samples and CD34+ HSPC in CBUs using the experimental design developed with KG1

cells. Autophagy flux levels were checked before and after cryopreservation and post thaw viability analysis was conducted using flow cytometry. The impact on cell potency post thaw was measured using CFU and pSTAT5 assay in CBUs from 3 separate donors.

Results obtained with fresh CB TNCs samples demonstrated that autophagic flux levels were moderately increased by autophagy activators (rapamycin MFI=228, trehalose MFI=279) and as previously seen no significant impact was observed in the presence of autophagy inhibitors when compared with untreated cultured cells pre freeze (control MFI= 180) (Figure 21A). Similarly, addition of DMSO and CPP to CB induced small increases in autophagy but differences vs untreated samples were not significant (Figure 21 A).

Next, CB TNCs were cryopreserved with DMSO/CPP along with the autophagy activators/inhibitors (with DMSO only). Analyses of the autophagy flux levels post thaw revealed that the modulators had a lesser impact on autophagy flux levels in primary CB samples than in the immortalized cell line KG1 cells, with again activators failing to raise autophagy above untreated controls, and the inhibitors only causing minimal non-significant reductions in flux levels (Figure 21B). However, and as previously observed, the levels of autophagy flux were significantly superior in post thaw samples when compared to pre freeze levels; indeed, the autophagy flux levels were almost 2 folds greater post thaw (Figure 21C).

The impact of autophagy modulation on the viability and recovery of primary CB CD34+ cells within CB cells are presented in Figure 21 D and E, respectively. No significant differences were observed in regard to the proportions of viable, apoptotic and necrotic CD34+ cells immediately post thaw (Figure 21D). However, the net recoveries of viable CD34+ cells appeared to be reduced with the autophagy inhibitors (3-MA and bafilomycin A1) though the differences failed to be significant. Conversely, addition of

neither trehalose nor rapamycin improved the recovery of viable CD34+ cells post thaw (Figure 21E).

Next, CFU assay measurements showed that CB stem progenitor's potency was negatively affected by the inhibition of autophagy by 3-MA but not by bafilomycin A1 (Figure 21F). The net number of CFUs recovered for CBU samples cryopreserved with the autophagy inhibitor 3-MA (2670±481) was significantly lower than with samples frozen with the autophagy the activator trehalose (15800±2451, Figure 21F left panel). Finally, addition of autophagy activators failed to improve CFU numbers (Figure 21F). Data obtained in CFU assay was corroborated with the IL-3-pSTAT5 activation assay. Results from this assay showed that with 3-MA, a significant reduction was observed in the recovery of functional CB CD34+ cells post thaw in comparison to control CB samples or trehalose samples (Figure 21F right panel). Putting it all together, it was observed that the level of inductions or inhibitions were lower in thawed CB TNC samples than what was observed in KG1 cells, but the impact of the autophagy modulators were similar between both the types of cells. Importantly, my results confirm activation of autophagy in the thawing process, and that early inhibition of autophagy with 3-MA results in significant reduction in cell viability and cell potency, whereas late inhibition with bafilomycin A1 only induces modest reductions.

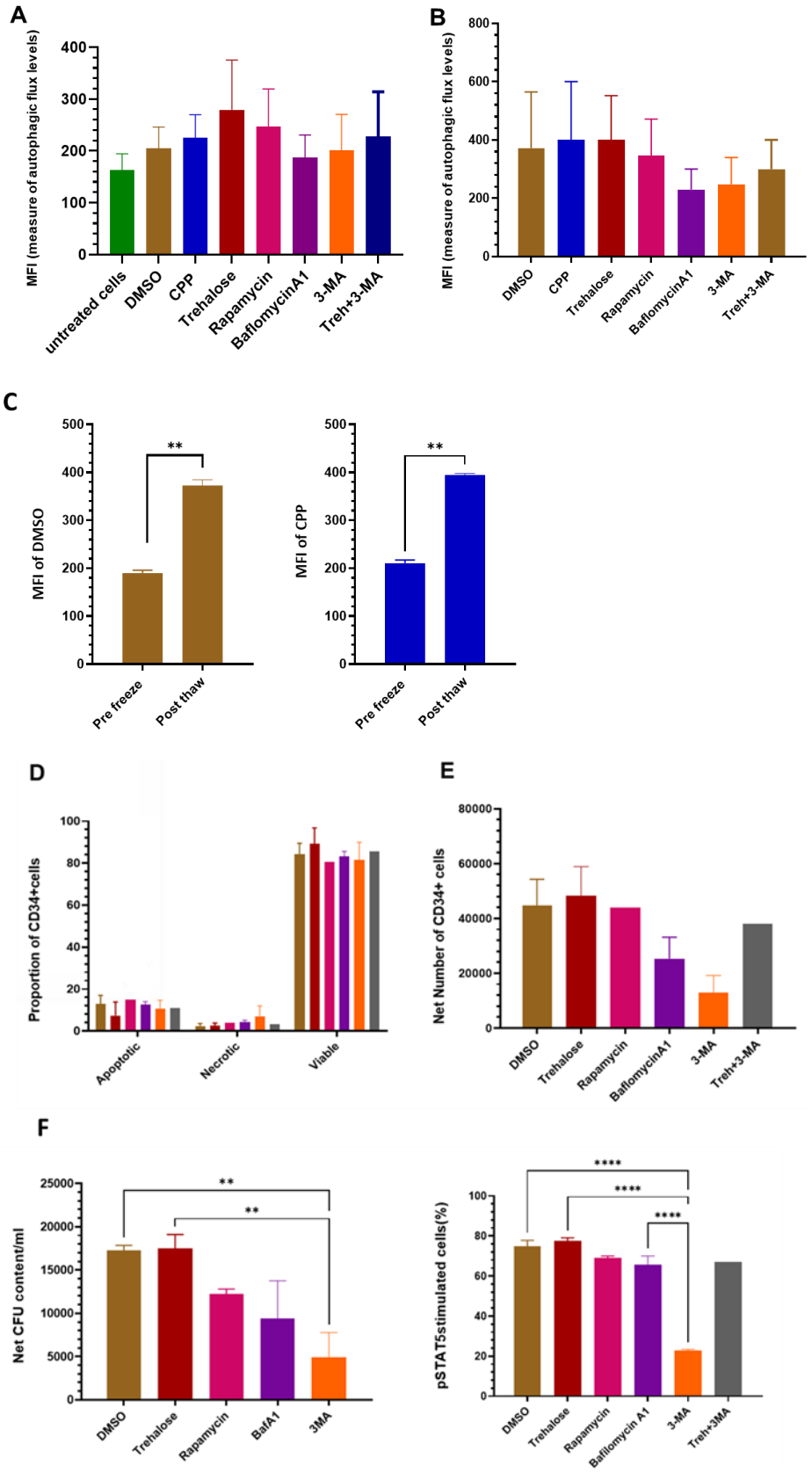


Figure 21: Impact of autophagy modulators on the cryoprotective properties of CB HSPCs.

(A) Autophagic Flux detection pre freeze using Cyto-ID dye. (B) Autophagic flux detection post thaw using Cyto-ID dye. (C) Comparison of autophagic flux levels in DMSO and CPP samples pre freeze and post thaw. (D) Viability measured with annexinV/Sytox dyes. (E) Recovery of viable CD34+ cells presented as cells/μL. (F) Net Number of CFUs recovered post thaw (left panel) Percentages of phospho-STAT5 CD34+cells to confirm the differentiation and proliferation capacity of CB cells (right panel). For panel C-F, rapamycin was tested once. Statistical significance was determined using paired parametric t-test, Data represent the mean +/- SEM. n=3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

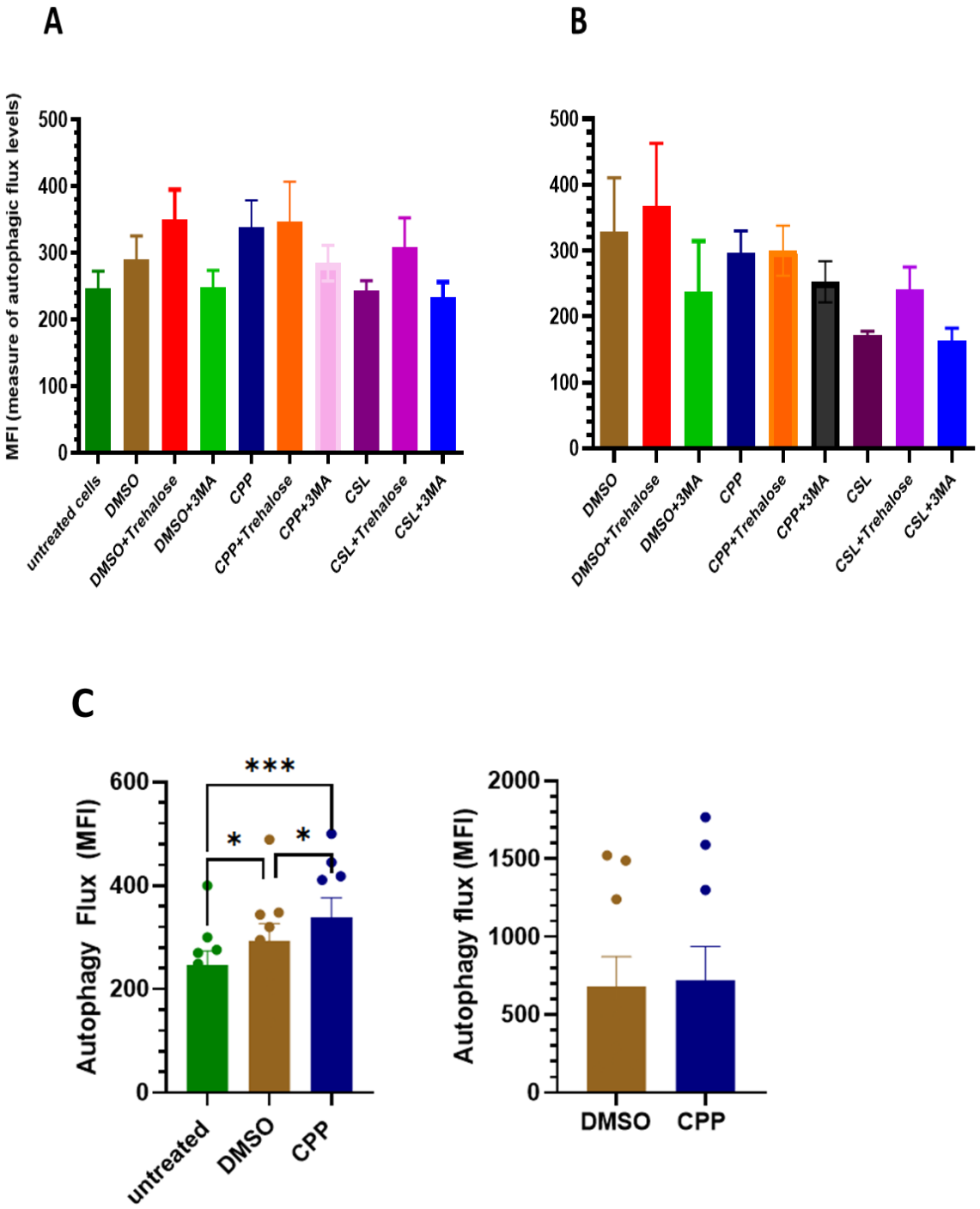
4.4.4. Impact of different CPAs on autophagy

Experiments were conducted to further examine the role of autophagy in the cryopreservation of CB HSPCs and the impact of CPAs on autophagy. To focus the work on HSPC enriched cells, pre expanded CD34+ cells were used for this work. Expanded HSPCs were treated or not with the autophagy modulators trehalose or 3-MA and cryopreserved with either DMSO, CPP or CSL.

Autophagy flux levels in CD34+ cell samples containing the 3 different CPAs were not significantly different to the control basal condition (Figure 22A) though two trends were observed. Indeed, a tendency for autophagy to be slightly elevated in CPP samples and lower in CSL samples were observed pre freeze and post thaw when compared to untreated samples and/or other CPAs. (Figure 22A-B) These observations will however need to be confirmed with additional samples. Also, a slight rise in autophagy was observed in DMSO and CSL samples following addition of trehalose though the differences failed to be significant when compared to their respective baseline condition. Addition of trehalose to CPP samples had no impact on autophagy flux. Addition of 3-MA had no significant impact pre freeze as previously observed with KG1 and CB cells pre freeze (Figure 22A). Moreover, both modulators failed to significantly impact autophagy flux level post thaw at least at the time of measurement.

As discussed above, levels of autophagy tended to be higher in CPP samples and perhaps DMSO in KG1, CB cells and CD34+ cells when compared to untreated samples, however the differences failed to be significant. To investigate this observation further, I pooled the autophagy flux data from all samples tested with DMSO and CPP (KG1, CB cells and CD34+ cells) to increase the statistical power. This analysis revealed that the addition of the two CPAs, DMSO and CPP, to cells was associated with significant increases in autophagy flux with CPP inducing the highest level (Fig 22C). However, the difference between DMSO and CPP failed to be significant post thaw (Fig. 22C).

Next, I measured the impact of autophagy modulation on the recovery of viable CD34+ cells post thaw using the ISHAGE methodology. This analysis confirmed a significant reduction in the concentration of viable CD34+ cells in the presence of 3-MA in DMSO cryopreserved samples (Figure 22D). Similar reductions in were detected with CPP and CSL samples though the differences did not reach statistical significances (Figure 22D). In contrast, addition of trehalose had no significant impact. Together, this data reveal that CPA may have an impact (CPP and CSL) on autophagy and that recovery of HSPC post thaw is reduced if autophagy activation post thaw is prevented regardless of the type of CPA used.


A
MFI (measure of autophagic flux levels)
500
400
300
200
100
0
untreated cells
DMSO
DMSO+Trehalose
DMSO+3MA
CPP
CPP+Trehalose
CPP+3MA
CSL
CSL+Trehalose
CSL+3MA
B
500
400
300
200
100
0
DMSO
DMSO+Trehalose
DMSO+3MA
CPP
CPP+Trehalose
CPP+3MA
CSL
CSL+Trehalose
CSL+3MA
C
Autophagy Flux (MFI)
600
400
200
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untreated
DMSO
CPP
Autophagy flux (MFI)
2000
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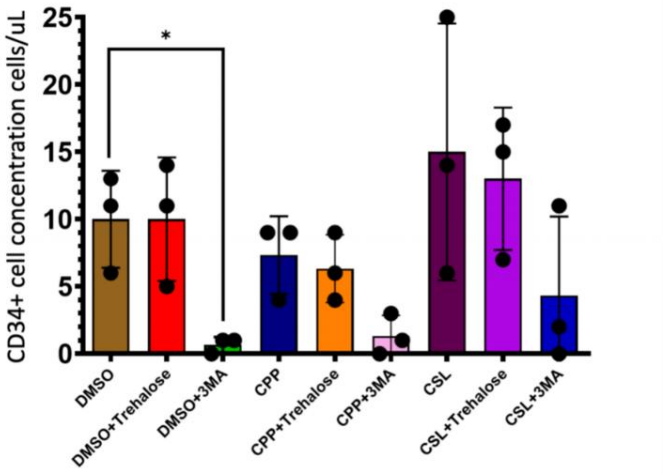

Figure 22: Impact of autophagy modulators and CPAs on HSPCs.

(A) Autophagy flux levels of all 9 conditions pre-freeze. (B) Autophagy flux levels of the experimental conditions post thaw. Two-way ANOVA was done. (C) Analysis of autophagy flux levels in untreated cells or cells incubated with DMSO or CPP pre freeze and post thaw. Mean ±SEM shown, n=9. paired parametric t-test, *p<0.05, ***p<0.01 (D) Recovery of viable CD34+ cells presented as cells/µL. Mean ±SEM shown, n=3. Statistical significance was determined using unpaired parametric t-test, *p<0.05.

4.4.5. Investigating the impact of CPAs on the expression of autophagy related genes in HSPCs

The effect of CPAs on the expression of key genes that mediate autophagy was examined by RT-qPCR assay by comparing HSPCs cryopreserved using the different CPAs. Fresh CB CD34+ cells that were never frozen were expanded in cultures and used as positive controls. The gene expression data showed that autophagy genes were highly expressed in freshly cultured CD34+ cells (Figure 23) and lower in thawed and expanded CD34+ samples. Within those, there was a noted reduction in the expression of autophagy genes in the CSL samples followed by DMSO cryopreserved HSPCs. The reduction seen in CSL samples further support the reduction in autophagy flux previously detected in CB cells (Figure 23B). Conversely, CPP had the highest level of autophagy gene expression of the cryopreserved samples tested (Figure 23).

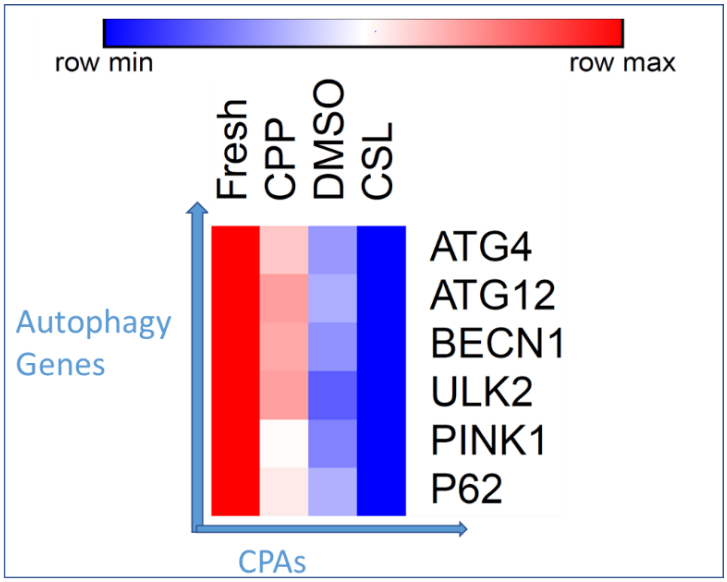

Figure 23: Heat map showing the impact of different CPAs on autophagy genes.

HSPCs were cryopreserved with different CPAs and RT-qPCR was performed post thaw. Mean of 3 triplicates for each sample. n=3

4.5. Discussion

In chapter 3, I studied the impact of different CPAs on the key biochemical mechanisms important for the survival of CB HSPCs, namely on bioenergetics and on cell membrane damage and integrity. In the present chapter, I tested the hypothesis that modulation of autophagy can impact viability and potency of CB HPSCs post cryopreservation. Toward this, I investigated the impact of different CPAs on autophagy and the effect of the autophagy modulation on post thaw CB HSPC' survival and potency.

Autophagy plays an important role in various aspects of hematopoiesis, such as HSC survival, differentiation, and function[177,206]. Dysregulation of autophagy can affect the balance between HSC proliferation and apoptosis which may lead to the development of hematological disorders[192,241]. The impact of cryopreservation on autophagy in mammalian cells is not fully understood. In fact, there is no information available regarding the role of autophagy In HSPC's survival following cryopreservation. Therefore, understanding the impact of cryopreservation on autophagy in HSPC biology is of significant value[202].

One the first finding of my work was that autophagy flux levels are significantly increased up to 2-fold in hematopoietic cells including CB HSPC following cryopreservation and thawing. In line with my work, a study by Bresciani *et al.* reported that autophagy was upregulated in kidney HEK cells after two freeze-thaw cycles, as measured by increased levels of LC3B-II and decreased levels of p62, two markers of autophagic flux[242]. Another study done by Gao *et al.* in 2017, reported that autophagy was upregulated in immature oocytes during vitrification-warming and in vitro maturation, and that inhibiting autophagy activated apoptosis via caspase-9 and -12 pathway[243]. The rise in autophagy seen in HSPCs post thaw is certainly consistent with the notion that autophagy is induced when cells are exposed to stress.

To study the functional contribution of autophagy on the post thaw survival and potency of HSPC, I exposed KG1 cells and CB HSPC to autophagy modulators. These included two activators in rapamycin and trehalose and, two inhibitors in bafilomycin A1 and 3-MA. Modulation of autophagy was optimized using KG1 cells and the autophagy flux assay (data not presented). Trehalose and rapamycin treatments raised autophagy flux levels pre freeze while the inhibitors reduced autophagy activation post thaw. These results set the stage for the analysis of the importance of autophagy in post thaw cell survival.

My data post thaw revealed that autophagy plays a vital role during cryopreservation since early autophagy inhibition before freezing resulted in significant detrimental effects post thaw. This study, to my knowledge provides the first evidence that autophagy modulation has an influence on the viability and potency of HSPCs post thaw. Indeed, early autophagy inhibition through 3-MA significantly reduced the recovery of viable KG1 cells and CB HSPCs. Moreover, 3-MA also induced a significant decrease in CFU numbers and in pSTAT5 positive cells HSPCs in cultures. Moreover, reduced recovery of CD34+ HSPCs following 3-MA treatment was observed independently of CPA used. These results

further support the conclusion that early blockade of autophagy through 3-MA significantly reduced the viability and potency of CB HSPCs post thaw. In line with my results, Salemi *et al.* in 2012, showed that in the presence of 3-MA the formation of autophagosomes was inhibited which resulted in decreased HSPC recovery (flow cytometric analysis), and potency (CFU analysis) of adult human HSPCs[194]. Moreover, Nomura *et al.* in 2021 demonstrated that autophagy is critical for HSC protection against harsh environments in the early neonatal stage and that autophagy deficiency impairs HSC self-renewal and engraftment due to oxidative stress and DNA damage[197].

However, late onset inhibition of autophagy with bafilomycin A1 did not induce such pronounced detrimental effects. This may be because as a late-stage autophagy inhibitor autophagy vacuoles may have already been accumulated in KG1cells and HSPCs thereby providing minimal levels of autophagy for cell survival. The autophagy initiation and elongation steps are very critical and govern the efficacy of the autophagy pathway. However, bafilomycin A1 did induce small reductions in the potency (CFU and pSTAT5 assays) of CB cells but the effects were not significant at this time. Likely additional CBU need to be tested. On the other hand, my results showed that CB HSPCs pre-treated and cryopreserved with trehalose showed minimal to no difference in autophagy flux levels post thaw compared to the baseline conditions. However, trehalose did increase autophagy levels pre freeze though differences with untreated cells were not always significant due in part to normal biological variation between human samples. Addition of trehalose pre freeze had no beneficial impact on viability and potency. This outcome of trehalose treatment was unexpected. This may be because autophagy is strongly induced post thaw (as shown herein), and that the trehalose induction of autophagy is insignificant compared to the induction induced by the freeze and thaw cycle. This result also contrasts to that of Martinetti *et al*. in 2017, who showed that supplementation of DMSO with trehalose

improved cell survival in thawed PBSCs[238]. This could be due to the difference in trehalose concentration used (up to 50-times greater when trehalose is used for CPA purposes), as well as the difference in experimental protocol. Moreover, while trehalose has been investigated as a CPA [127,130], these studies did not investigate if autophagy modulation was involved or not in the protective effect of trehalose.

Next, I tested for the first time to my knowledge the impact of different CPAs (DMSO, CPP and CSL) on autophagy in primary CB HSPCs. I selected these 3 CPAs since I had identified CPP and CSL as valuable alternatives to DMSO. Addition of CSL had the least impact on autophagy pre freeze, while addition of CPP and DMSO slightly increased autophagy flux. However, as previously observed with KG1 and CB cells, the differences in autophagy flux failed to be significant when compared to untreated samples. As such, I performed additional analysis by pooling the autophagy flux levels from all samples used (KG1, CB TNCs, CD34+ enriched) treated or not with DMSO and CPP. This statistical analysis revealed that autophagy flux levels were significantly induced by both CPAs and significantly more by CPP than DMSO in cells pre freeze These observations suggest that CPAs may have a different impact on autophagy with some triggering autophagy in HSPCs.

Next, I measured the expression levels of autophagy-related genes by RT-qPCR to further study the impact of these CPAs on this pathway. Interestingly, the data revealed that the expression of autophagy-related genes in HSPCs post thaw was at the highest in CPP-protected samples and lowest in CSL samples. These results corroborate the trends observed in autophagy flux analysis in CSL and CPP CD34+ samples, where autophagy flux was slightly higher and lower in CPP and CSL samples, respectively. Whether these effects govern in part or not the efficacy of these CPAs with CB HSPCs and how they affect autophagy is unclear at this time and will need to be investigated in future work.

There are several limitations to the present work that I acknowledge; first, the low number of units tested due to time constraint and a shortage of CBUs at the time of my work. It would be beneficial to increase the number of CBUs tested to acquire more robust results. The second limitation is that the optimization of autophagy modulators was done using KG1 cells instead of CB cells. The different nature of these cells might have an impact on the efficacy of autophagy modulation. Moreover, I did not explore potential changes at the protein level of key proteins implicated in autophagy due to time constraints.

In conclusion, the results obtained demonstrate that cryopreservation leads to an induction of autophagy in cryopreserved CB HSPCs. My data also demonstrated that early autophagy inhibition resulted in significant reduction in recovery of viable and functional CB HSPCs stressing the importance of autophagy post thaw. Lastly, my results on 3 distinct CPAs suggest that those may have different impact on autophagy in CB HSPCs, but these results remain to be confirmed with additional samples. Further studies are also needed to elucidate whether autophagy modulation could improve the viability and potency of HSPCs post thaw.

4.6. Acknowledgements

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CHAPTER 5

GENERAL DISCUSSION[2]

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Clinical Trial

Current and Future Perspectives for the Cryopreservation of Cord Blood Stem Cells

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HSCT and recent cell therapies have led to the swift development of regenerative medicine in many areas, from basic transplantation to chimeric antigen receptor-based immunotherapies and innovative gene editing therapies[182]. However, most cell therapies are dependent on the efficient storage of, and cryopreservation remains the most useful way to maintain quality of the product over long duration of storage and shipping[167].

HSPCs from sources like PBSC, CB and BM cells are often cryopreserved before use. CB grafts have demonstrated to preserve high post thaw cell viability and engraftment activities when properly cryopreserved for periods greater than 15 years[9]. However, clinical studies have shown that loss of cell viability post thaw decreased the potency of HSCT and reduced product efficiency and poor quality of engraftment[31]. The most substantial source of cell loss post thaw is due to necrosis followed by apoptosis [244]. These events are a direct result of accumulating cryoinjuries derived from dehydration, imbalance in osmotic stress and uncontrolled growth of ice (i.e., ice recrystallization) that happen during the process of cryopreservation. Cryopreservation can deteriorate the graft quality if it is poorly coordinated. In fact, cryopreservation and thawing of cells exacerbate cell lesions leading to apoptosis and necrosis[168]

Prominently, reduction in potency linked to poor cryopreservation and decreased cell function is associated with risk of graft failure[61]. Therefore, it is extremely important for cryopreservation to be done following optimized standard operating procedures in banks and in clinics. Equally important is for continuous research and development in cryobiology to better understand this process, identify new routes of optimization to ultimately improved the cryopreservation process.

CPAs play an essential role in minimizing cryoinjuries and in determining the success rate of cryopreservation. As indicated in Chapter 1, new CPAs have been identified but their cryoprotective properties and mechanism of action are not always well studied[148–

[151,218]. This is especially relevant in HSPC grafts, where DMSO has been used for decades as standard CPA. However, it is possible that new CPAs may be as good if not superior to DMSO for the cryopreservation of HSPCs, but this possibility remained unaddressed at the time. While the exact make up of those solutions is not fully disclosed, it is still important to assess their usefulness in protecting stem cell grafts from cryoinjuries. Hence, in Aim 1 of my dissertation, I have conducted experiments to characterize the cryoprotective properties of new CPAs on CB cells and HSPCs (Chapter 2). Additionally, I investigated theIR impact on the proliferation and differentiation activities of cryopreserved HSPCs. Thereafter, I functionally validated the effect of selected CPAs on engraftment activity of HSC graft using mice xenotransplantation model.

In aim 1, I identified CPAs that effectively cryoprotected HSPCs while others failed to. Hence, in aim 2, I investigated how these CPAs impact key biochemical pathways known to be essential for cell survival (Chapter 3). Recent evidence suggests that biological mechanisms such as glycolysis and mitochondrial respiration may be affected during cryopreservation[155]. For instance, a recent study revealed that cryopreservation of primary immune cells altered their metabolism in a time-dependent manner, as suggested by enhanced glycolytic activity and decreased aerobic respiration[152]. However, the role of these pathways in HSCs cryobiology is poorly understood at the present time, in this aim, I sought to investigate the impact of CPAs on mitochondrial respiratory pathway and glycolysis cycle in HSPCs in context of cryobiology. [245]Moreover, we know that necrosis is a major source of cell loss during cryopreservation[73]. As such, it is important to characterize the impact that CPAs have on HSPC' cell membrane characteristics such as integrity and damages. Therefore, in this aim, I also studied the short-term effect of these CPAs on hematopoietic cell membrane before freezing.

Several studies have now revealed that autophagy is important for the regulation and function of HSPCs[77,161,180,185] .Furthermore, it has been shown that autophagy plays a pivotal role in protecting adult HSCs aiding in tolerating adverse conditions that naturally occur in bone marrow environment[205]. However, very few studies have addressed the role of autophagy in HSPCs cryopreservation [26]and, the impact that CPAs may have on it. Thus, to complement my findings on the characterization of new CPAs in HSPCs (Chapter 2) and on the impact that these CPAs have on bioenergetics and cell membrane of HSPCs (Chapter 3), I characterized the impact of CPAs and cryopreservation on autophagy in HSPC (Chapter 4). I also conducted experimentation to define the importance of autophagy in the cryopreservation of HSPCs.

5.1. Role of CPAs in cryopreservation

CPAs are imperative to limit the damage to cells during the freezing and thawing processes that would otherwise reduce cell viability and function during cryopreservation[39] [42]. The majority of CPAs act by interacting one way or another with water molecules, thus, altering phase transition within cell membranes and organelles[61]. DMSO is the standard intracellular CPA used for HSC grafts[133]. It permeates cell membranes and protects cells from cryoinjury associated with slow cooling rates by preserving the cell and reducing "solution effects"[84,244]

The identification of new CPAs (example- trehalose[127,128,130,213,236,238]) has facilitated the development of new commercial solutions such as CryoStor (Bio Life Solutions), which can be used with lower DMSO concentration with HSC grafts[148,245]. It has been recently demonstrated that slow addition of CryoStor led to better post thaw recovery of CB CD34+ cells[148]. Moreover, other development has resulted in new CPA solutions entirely devoid of DMSO. For instance, a xenotransplant study in 2018 revealed that human engraftment was retained in PIM-protected PBSC grafts[150]. To my knowledge, that work has

demonstrated for the first time that HSC graft could be cryopreserved without DMSO but still maintain high engraftment activity. Subsequently, several other DMSO-free solutions have become commercially available, such as CryoNovo, CryoScarLess, StemcellKeep and CryoProtectPure. But these CPAs have never been tested for HSPC grafts. So, it would be beneficial to evaluate their efficacy in cryoprotection of HSPC grafts.

Thus, I investigated whether new these new CPAs could provide adequate cryoprotection to CB HSPC grafts. For this, four different cryopreservation solutions CPP, CSL, CN and PIM were compared to DMSO used as standard benchmark control. The results revealed that CPP provided the best recovery of viable CD45+ and CD34+ cells post thaw, followed closely by CSL. CPP and CSL were also shown to retain high potency CB HSPCs and progenitors.

The lowest recovery of viable cells and lowest potency were seen with CN, which was the direct result of a high level of necrosis. The most reasonable explanation for this result is the excessive formation of intracellular ice during freezing and/or ice recrystallization post thaw. CN was originally derived from MSCs, which differ in many ways from HSCs in size and cell membrane composition (Atlantisbioscience.com). This clearly demonstrates the cell type dependency of CPA's cryoprotective characteristics. Likewise, PIM supplemented with 2% human albumin did not match the recovery of viable CD45+ and CD34+ cells or potency seen with the DMSO control, which we had expected based on previous reports[149,218]. The recovery of clonogenic progenitors was also lower with PIM. The annexin V/Sytox viability results revealed that as a stand-alone CPA with albumin, PIM fails to protect HSCs leading to cell membrane rupture likely due to excessive intracellular ice formation. However, when reviewing their original work, no data was presented on the net recovery of viable cells and other data was normalized in some assays per viable cells, which may have masked the lowest cell recovery[149].

Moreover, recent work from the manufacturer of PIM lent support to my results, since their follow up study also ruled out PIM as a sole CPA at least for adipose-derived stromal/stem cells[119].

Furthermore, the engraftment activity of DMSO and CPP-cryopreserved CB buffy coat grafts was investigated using the NSG mouse transplant model. The results of the two transplant experiments confirmed that both DMSO and CPP provided sufficient cryoprotection of CB grafts cells and preserved the engraftment activity of CB HSPCs. This was evident by the high levels of human platelets and lymph myeloid leukocytes throughout the follow-up period and by the high level of long-term human BM engraftment in CPP recipients. Future work will be needed to expand my finding by testing additional donors and performing secondary transplants to provide assurance that long-term HSCs are adequately protected.

Originally, CPP was reported to be a good cryopreservation solution for adherent cells (www.aicryo.com), but this did not guarantee that it would be equally suitable for HSCs. As was discussed in the paragraph above, the efficiency of any CPA depends significantly based on the cell type. This is in part due to differences in membrane composition, resulting in diverse types of resistance to changes in osmotic pressure and permeability to water and solutes, which can lead to different post thaw outcomes[113,116,167 246]. The balanced salt-based formulation of CPP contains glycol derivatives, with individual concentrations conforming to Good Manufacturing Practice standards, as indicated by the US Food and Drug Administration's Code of Federal Regulations Title 21. The serum-free formulation contains non-toxic polymeric protein components. The specific formulation is regarded as a trade secret by the manufacturer.

An optimal technique for the cryopreservation of any cell type needs the consideration of many factors including the composition of the CPA solution, freezing rate,

storage temperature, and post thaw processing to name a few. All these variables come together to prevent cryoinjuries that otherwise lead to loss of viability and function. The successful development of optimized CPA solutions will denote a substantial enhancement for the cryopreservation of HSC grafts and for the field of cellular therapy.

In my aim 1, I successfully identified CPP and CSL as CPA solutions of interest that efficiently protected HSPCs and others like CN and PIM that did not. My next line of investigations was set to understand the impact that the CPAs have on HPSCs key biological processes to understand why that some CPAs worked well and others not.

5.2. Impacts of CPAs on HSPCs bioenergetics pathways and cell membrane

Mitochondria plays an essential role in HSC maintenance and their ability to differentiate. The function and shape of HSC's mitochondria can be modified due to cryopreservation which can lead to a rise in the formation of ROS, impairment of ATP synthesis, and surge in apoptosis[156]. The capability of HSCs to engraft and endure long-term repopulation following transplantation may be modified by these alterations[188]. To my knowledge, the impact of CPAs on CB HSPCs bioenergetic pathways has not been investigated in depth. Of note, recent research suggests a strong correlation between cryopreservation of stem cells and respiratory pathways[156,158,221]. Hence, I sought to fill this knowledge gap about the impact of CPAs on bioenergetic mechanisms in HSPCs.

I have utilized the seahorse assay to measure the changes in bioenergetic characteristics of HSPCs cryopreserved with DMSO or CPP (Chapter 3). I had to focus my investigation on these two CPAs since access to the Seahorse apparatus was limited and at as was the source of HSPC for this work. Interestingly, my results revealed that DMSO cryopreserved HSPCs had significantly higher OCR and ECAR activity when compared to CPP cryopreserved HSPCs. This change was observed in HSPC expanded post thaw

revealing that the impact of DMSO persisted at least a week. However, HSPCs frozen with either CPAs responded in a similar fashion to the sequential addition of the drugs during the assays as revealed by the parallel curves obtained with DMSO- or CPP- HSPCs in both OCR and ECAR responses. This implies that these HSPCs cryopreserved with DMSO have a higher maximal activity than CPP cryopreserved cells and a stronger supply of energy from both TCA cycle and glycolytic pathway. This phenomenon could be explained by the impact of DMSO on glycolytic genes as discussed below, which may itself arise from the modulation of epigenetic markers by DMSO[169]. Another possibility that is yet to be tested is that CPP reduced the OCR and glycolytic capacities of the HSPCs. Further work using fresh HSPC and perhaps CSL-cryopreserved HSPCs could help narrow down the impact of these CPAs on HSPCs.

I complemented the seahorse functional data with a gene expression analysis of selected glycolytic genes. The former was selected based on the amount of evidence linking glycolysis to HSC biology[154,155]. The RT-qPCR data obtained indicated that of the three CPAs evaluated, DMSO-protected HSPCs had the highest level of expression of the glycolytic genes, superior to those seen with CPP and CSL. The difference between DMSO and CPP supports the greater level of glycolytic activity in DMSO-HSPCs detected by seahorse assay. In line with this, a study by Chen *et al.,* revealed that the DMSO induced a shift from oxidative phosphorylation to glycolysis in HSPCs by increasing the expression of glycolytic genes as observed in mouse BM- HSPCs[169]. Also, a study conducted using mononuclear cells revealed that DMSO present during cryopreservation was moderately protective for mitochondrial substrate-linked oxygen consumption; though, dilution from DMSO caused some variations in mitochondrial function[247]. Another study investigated the metabolic profiles of pre-frozen and frozen sperm cells. The results indicated that

DMSO treatment increased the expression of glycolytic genes, as well as the production of lactate and ATP[155].

Loss in membrane integrity leads to necrosis, which is the main mechanism in loss of viability during the freezing and thawing cycle[162]. In Chapter 2, I showed that some CPA failed to protect CB HSPC from cryo-induced necrosis, with CN and PIM showing pronounced increase in necrotic CB cells post thaw[226]. Consequently, the impact on cellular membrane integrity independently of freezing and thawing in HSPCs was investigated using two complementary assays. Results from the LDH assay revealed that there were no significant differences in cellular membrane leakiness between HSPC samples exposed for 30 mins to the different CPAs in comparison to untreated control cells. Likewise, LP results indicated a similar trend between the cell membrane oxidative stress levels of HSPCs exposed to the same CPAs. The lack of differences in CD34+ HSPCs between the different CPAs indicate that the differences in CD34+ potency cryopreserved with the same CPAs observed in Chapter 2 were not due to a short-term physical damage and toxicity inflicted by the CPAs on the HSPCs before cryopreservation.

In conclusion, I showed that CPAs can have a direct impact on the bioenergetic pathways of HSPCs and that the three CPAs tested had minimal impact on cell membrane integrity and lipid peroxidation levels pre freeze.

5.3. Impact of cryopreservation and CPAs on autophagy

Autophagy is a highly conserved cellular process by which cytoplasmic components are sequestered in autophagosomes and delivered to lysosomes for degradation. Recent literature has confirmed that autophagy plays a crucial role in the survival and function of HSCs[26,32,179,185,231] . However, no studies have investigated the role of autophagy in cryopreserved HSPCs to date. Hence, I have investigated in my Chapter 4

the impact of cryopreservation and CPAs on the process of autophagy in HSPCs and evaluated the influence of autophagy modulation in CB HSPCs.

Importantly, my results confirmed activation of autophagy in HSPCs during and/or after the thawing process. These findings with induction of autophagy in other cell type such as the kidney HEK cells after two freeze-thaw cycles, as measured by increased levels of LC3B-II and decreased levels of p62, two markers of autophagic flux[242]. Another study reported that autophagy was upregulated in immature oocytes during vitrification-warming and in vitro maturation, and that inhibiting autophagy activated apoptosis[243]. Interestingly, a study by Qiu *et al.,* stated that human HSCs with low mitochondrial activity[248], is indicative of high autophagy levels, exhibited greater maintenance of stem cell potential ex vivo and superior long-term engraftment capacity in immunodeficient mice compared to human HSCs with high mitochondrial activity and lower autophagy levels[12]. The authors suggested that low mitochondrial activity and high autophagy levels could be used as markers to select the most potent human HSCs for cryopreservation and transplantation[12,25].

More importantly, my work also revealed that early inhibition of autophagy with 3-MA led to a significant reduction in cell viability and cell potency of CB HSPCs from CBU post thaw. In line with my results, Salemi *et al.,* showed in adult HSPCs that in cultures in the presence of 3-MA the formation of autophagosomes was inhibited which resulted in decreased recovery (flow cytometric analysis), and potency (CFU analysis)[194]. Another study by Yuan *et al.,* revealed that bafilomycin A1 blocks autophagic flux leading to the accumulation of p62 and LC3-II in acute lymphoblastic leukemia cells[239]. Moreover, Nomura *et al.,* demonstrated that in the early neonatal stage autophagy deficiency impairs HSC self-renewal and engraftment due to oxidative stress and DNA damage[197]. Altogether,

these results provided more information about the role of autophagy in cryopreservation of hematopoietic cells.

Next to investigate the process of autophagy in cryopreservation further, I measured the autophagy flux and post thaw viability using different CPAs in combination with autophagy modulators in CD34+ enriched cells. Included were the DMSO-free CPAs which provided similar protection and efficacy to HSPC than DMSO[226]. The data confirmed that inhibition of autophagy with 3-MA leads to loss of function and HPSC number post thaw irrespective of the CPA used. This investigation also revealed that HSPCs exposed to CPP had a higher basal level of autophagy, followed by those preserved with DMSO, whereas CSL had almost no impact when compared with untreated control, though the differences were not significant. However, further analysis of all autophagy flux pooled data obtained with fresh cells exposed to DMSO and CPP showed that addition of both CPP and DMSO to hematopoietic cells induces a rise in autophagy flux, and significantly greater with CPP. Additionally, RT-qPCR data revealed that the expression of autophagy-related genes in HSPCs was highest in CPP-protected samples and lowest in CSL samples. These qPCR results corroborate the two trends observed in autophagy flux analysis in CPP and CSL HSPC samples.

Taken together, my results confirm the strong activation of autophagy in HSPCs post thaw and demonstrated for the first time that inhibition of autophagy prevents the cells from activating pathways as a reaction to the stress of freeze/thaw cycle in cryopreserved CB HSPCs. This study has also investigated for the first time the impact of different CPAs on autophagy and if modulation of autophagy could steer the efficacy of cryopreservation of CB HSPCs. The data suggested that the tested CPAs have differential impact on autophagy. Although future research is required to better elucidate whether autophagy modulation could improve efficacy of cryopreserved HSPCs.

CHAPTER 6

FUTURE PERSPECTIVE

6.1. Follow up on cryoprotective properties of the tested CPAs

In my thesis, CPAs like CPP and CSL emerged as good CPAs for HSPCs. Moving forward, it would be beneficial to further investigate their cryoprotective properties on HSPCs in different settings and using more stringent techniques as discussed below.

Follow-up investigations are required to study the role of new emerging CPAs compared to conventional CPA for CB graft. For one, future studies should involve serial transplant assays to better characterize the capacity of CPP to cryoprotect HSCs with long-term engraftment and self-renewal capacity. Similar investigation could also be done with CSL. Moreover, it would be important to test these CPAs with other sources of HSC such as PBSC and BM. Conversely, one could also test whether supplementation of some of these CPAs with low concentration of DMSO could provide better cryopreservation outcomes.

One important challenge during cryopreservation is to prevent ice recrystallization, which can damage the cell membrane and reduce the viability of the preserved cells[121249]. Moreover, the influence of the tested CPAs on cryoinjuries normally associated with ice recrystallization during freezing and thawing processes in CB HSPCs is an area still open for investigation that would be interesting to explore in the near future. Previously our lab has investigated the capacity of 2 N-aryl-D-aldonamides molecules, IRI 2FA and IRI PMA, to improve the recovery of CB progenitors post-thaw and engraftment when used in complementation with DMSO[191]. In my opinion, this represents an excellent opportunity to investigate the capacities of the new CPAs like CPP and CSL to control ice recrystallization alone or in combination with different IRIs such as 2FA. This could be

assessed for their ability to inhibit ice recrystallization using the splat-cooling assay[250–252]. This method is used to evaluate the ice crystallization inhibition activity of CPAs and their impact on size of ice crystals[253].

Another important aspect that could not be studied in more detail in my thesis was the impact of CPAs on apoptosis during cryopreservation of HSPCs. My results shown in Chapter 2 revealed that HSPC cryopreserved with the different CPAs had different levels of apoptosis, such as highest with DMSO. The mechanism for this is unclear at this time. Apoptosis might be activated by one of two mechanisms, either intrinsic (mitochondrial pathway) or extrinsic pathways (death receptor like Fas, TNFR1)[243]. In the mitochondrial pathway, pro-apoptotic and protective molecules play a role to regulate mitochondrial permeability and it's activated by most cellular stresses[197]. On the other hand, in extrinsic pathway, death receptors are members of the TNF receptor family (TNFR1 receptor and TNF-related apoptosis-inducing ligand i.e., TRAIL) and related protein called Fas (CD95) contain a death domain which needs to be activated to execute apoptosis[229,234]. Thus, investigation of the impact of the tested CPAs on the expression levels and activation of pro and anti-apoptotic proteins in HSPCs would provide some insights on whether and how different CPAs may trigger apoptosis in HSPCs.

Transient warming events (TWEs) are described as transient exposures of a cryopreserved product to temperatures over a recommended critical storage temperature, such as -150 °C for CBUs (NetCord-FACT specifications)[90]. TWEs can cause ice recrystallization, which can permanently damage cells, such as the viability and potency of lymphocytes and CB-MSCs[254]. In a recent study conducted at our lab, internal core temperature and warming rates of CBUs were monitored and it was shown that TWE can take place during routine operational CB banking procedures, and that those can lead to decline in potency[159]. Indeed, another study measured an 8.5% reduction in CFU counts

for CBUs exposed to 10 consecutive TWEs to −80 °C [116]. Therefore, it would be fascinating to compare the capacity of the different CPAs used in my project to protect HSPCs from TWE-induced cryodamage and loss in potency using a validated TWE model. Moreover, it would be valuable to investigate the importance of autophagy in the context of TWE settings since one can expect cell stress to be greater under such settings.

Overall, these diverse approaches will provide further insights into the cryoprotective properties of new emerging CPAs that could perhaps one day be useful for the cryopreservation of HSPCs.

6.2. Elucidating the role of bioenergetics in HSPCs function during cryopreservation

It has been shown in previous literature that one of the key impacts of cryopreservation on primary immune cells is on bioenergetic pathways[152]. Therefore, it is very important to observe the impact of CPAs and cryopreservation on mitochondria and glycolytic pathways in HSCs by various techniques such as done in my thesis.

The seahorse data acquired in my work demonstrated that CPAs had a significant impact on bioenergetic mechanisms in CB HSPCs. DMSO cryopreserved HSPCs depicted higher OCR and glycolytic potential than CPP protected HSPCs, indicating higher ATP production capacity for DMSO HSPCs glycolysis and through mitochondrial respiratory pathway. To complement my seahorse data, one could further quantify mitochondrial content using electron microscopy and mitochondrial membrane potential monitoring, newly developed methods, such as mitochondrial ultrastructural determination, mtDNA mutation assays, metabolomics, and analyses of regulatory mechanisms, have also been utilized in recent years[220,255]. In addition, since I have tested only 2 CPAs in my thesis,

testing more CPAs would add more insights if CPAs can impact the bioenergetics of cryopreserved HSPCs.

In my study was that cryopreserved HSPCs were cultured for 7 days after thawing before the seahorse analysis was performed. It would be interesting in the future to determine whether the differences seen between CPP- and DMSO-HSPCs are maintained after prolonged periods, such as 2 to 3 weeks of culture. Even more fascinating, would be to perform the seahorse analysis on BM CD34+ HSPC isolated from CPP-HSCP and DMSO-HSPC transplanted mice. The latter would allow us to determine if the impact of CPAs on bioenergetics can extend up to 4-5 months. Moreover, further studies could investigate the direct impact of cryopreservation on bioenergetics of HSPCs without cell culture. In this approach, fresh CD34+ enriched HSPCs could be compared to thawed CD34+ HSPCs to evaluate the influence of cryopreservation and thawing on bioenergetic mechanisms.

As indicated above the differences observed in DMSO/CPP HSPCs bioenergetics were sustained even with 7 days cell culture. This indicates that this could be due to the impact of CPAs on epigenetics of HPSCs. Hence, bioinformatic analysis could be conducted to investigate the impact of different CPAs on epigenetic markers. Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) is a technique used to assess genome-wide chromatin accessibility. ATAC-Seq helps to uncover how chromatin packaging and other factors affect gene expression[256]. As such, ATAC-seq could be used for the analysis and comparison of the epigenome of HSPCs treated with different CPAs. Single-cell RNA-sequencing (scRNA-seq) could also be used in further investigating the impact of CPAs on epigenetic genes in HSPCs[89]. These bioinformatic tools would provide useful information for deciphering the impact of CPAs on epigenetics of HSPCs. The impact of the CPAs on epigenetics on genes that related to the bioenergetic pathways could

be fully characterized. Conversely, a simpler and quicker approach would be the use of intracellular FACS using antibodies that recognize epigenetic marks like histone modifications (acetylation and methylation) as well as DNA methylation itself[182]. This analysis could identify epigenetic marks that are significantly impacted by the different CPAs. Altogether, these analyses could aid in identifying genes and pathways that can be further studied in the future in functional studies using chemical inhibitors, gene knockdown or over expression studies for instance[35].

Another area worth pursuing is to further characterize the impact of DMSO and other CPAs on glycolysis. At least one previous study has shown that the expression of genes and proteins involved in glycolysis, such as HIF-1, AMP-activated protein kinase, mTOR, is altered in cryopreserved HSCs[166]. Further, to determine the level of glycolytic enzyme activity, ELISA or colorimetric assays could be used. Immunometabolic studies could be conducted using mass spectrometry or high-performance liquid chromatography to assess the concentration of glycolytic metabolites and examine the effect on HSCs[153]. Moreover, further investigation could be conducted to investigate the impact of cryopreservation on glycolysis in HSPCs using Cayman's Glycolysis Cell-based assay, a colorimetric method that detects L-lactate, the end-product of glycolysis, produced and secreted by cells[155].

A recent study raises the possibility that HSC cryopreservation may gain advantage from the use of glycolytic inhibitors[39]. Others have demonstrated that HSCs can be protected from cryoinjury post-thaw by lowering the formation of ROS, maintaining the mitochondrial membrane potential, and inhibiting apoptosis, glycolytic inhibitors including 2-deoxyglucose (2-DG) and 3-bromopyruvate (3-BP)[156]. In addition, it has been shown that glycolytic inhibitors can improve HSC homing and engraftment by controlling the expression of cell surface markers like integrins, CXCR4, and CD34[257]. Consequently, it

would be interesting to elucidate the role of these glycolytic inhibitors using different CPAs in cryopreservation of CB HSPCs.

6.3. Gene function studies to investigate the impact of CPAs and cryopreservation on autophagy pathway

My results in chapter 4 revealed that autophagy activation is crucial for the survival of HSPCs post thaw independently of the CPA used. Therefore, further investigation is vital to understand the impact of cryopreservation and different CPAs on the stages of autophagy pathway. One approach could be comparison of fresh HSPCs vs post thaw HSPCs using knockdown experiments of autophagy genes important for autophagy mechanisms such as ATG 7, ATG 9[185]. Targeting gene essential to autophagy would also ensure that the effects are specific to autophagy, since chemical inhibitors can also impact other pathways through non-specific actions. Targeting early and late stages gene of autophagy pathway would be interesting to complement my current findings. Future work could also involve the use of complementary techniques[258] to confirm activation of autophagy such as immunobloting of activated autophagy proteins (such as ULK1/2, ATG13, RB1CC1/FIP200, and ATG101), a gene reported GFP-LC3/p62 autophagy detection assay, LC3-II autophagy enrichment kits and finally and, live cell LC3 lentiviral fluorescent biosensors, to measure the autophagy levels in cells[242].

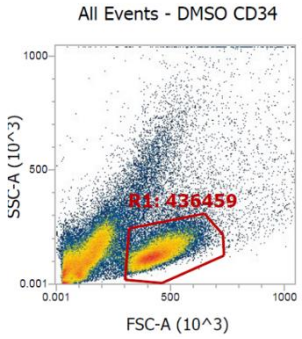
In my thesis, I have used a limited number of autophagy modulators (drugs) to investigate the impact of CPAs on autophagy in HSPCs. A follow up study could see a more extensive screening of autophagy modulators using autophagy small molecule library. This could be a useful tool to further characterize the role of autophagy in cryopreservation[183] and help identify stronger inducers of autophagy that may promote cell viability and potency of HSPCs post thaw.

CHAPTER 7

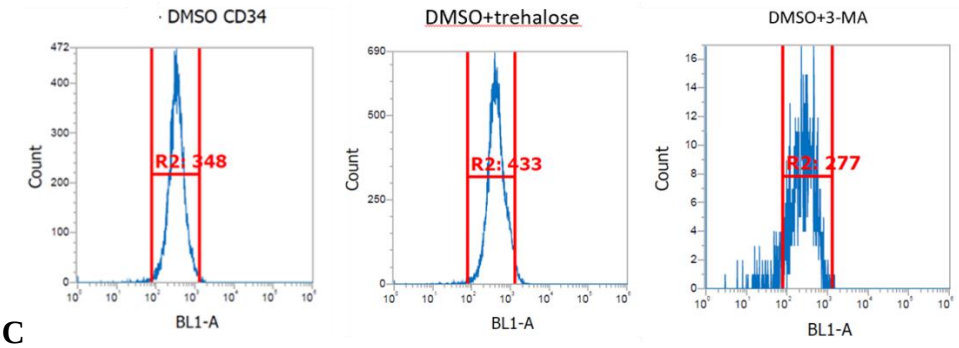
APPENDIX

7.1. Autophagy flux level analyses using different CPAs and autophagy modulators in CB HSPCs post thaw

A



B



C

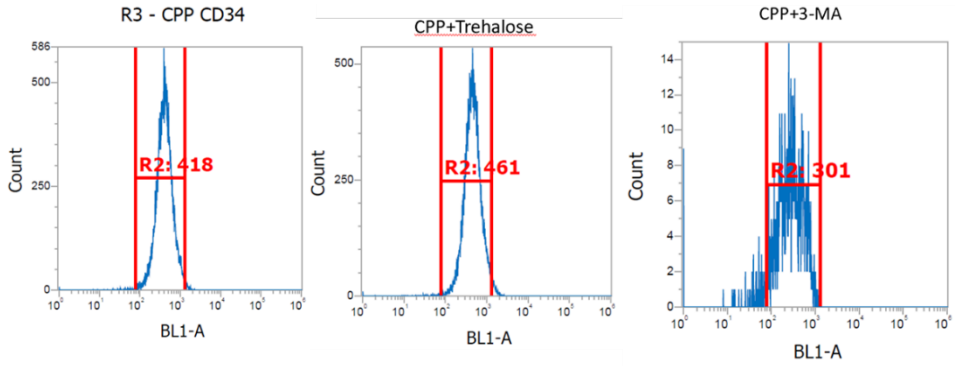


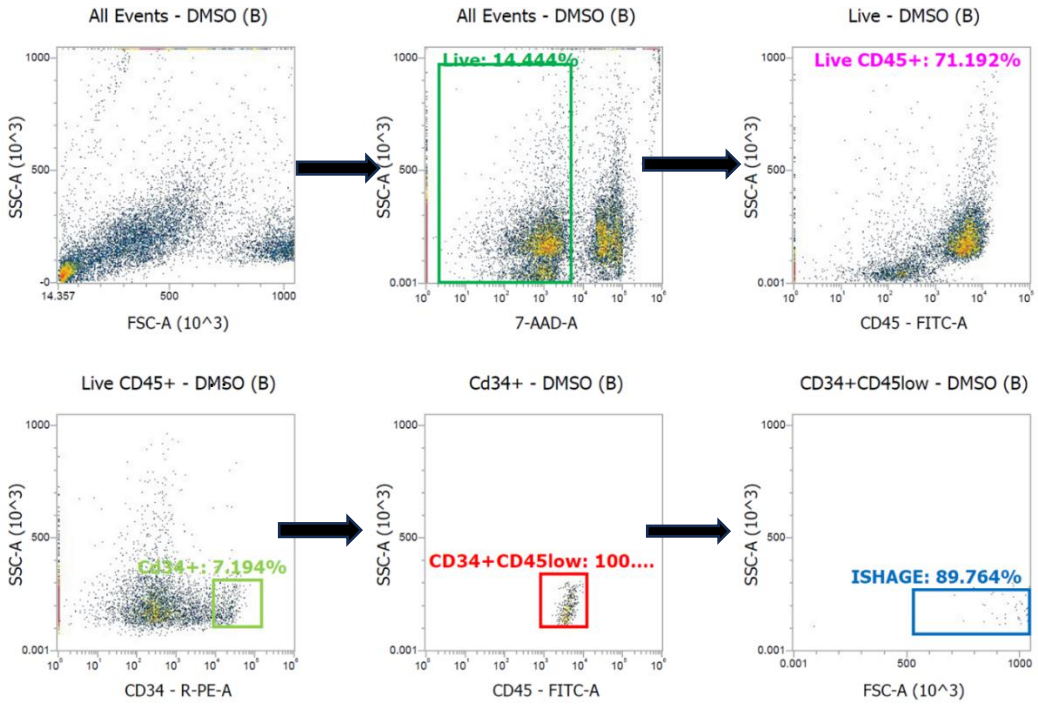
Figure 24: Impact of CPAs and autophagy modulators on autophagy flux levels of CB HSPCs post thaw.

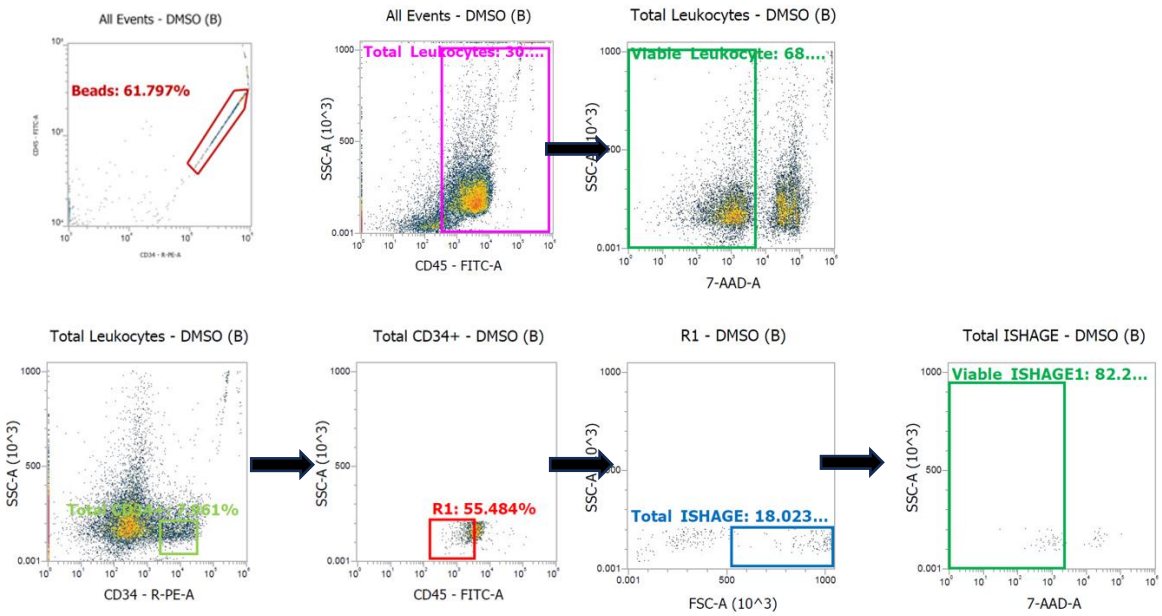
Expanded CD34+ cells were cryopreserved with the following conditions- CPA alone, CPA with autophagy activator and CPA with autophagy inhibitor. After thawing, the cells

were analyzed for autophagy flux levels with CYtoID. Live cells were gated by separating them from debris and then MFI was measured which was equivalent to the autophagy flux level of respective cell samples. (A) Representative autophagy flux level analysis of DMSO cryopreserved CD34+ cells illustrating the strategy followed. (B) Representative autophagy flux level analysis of DMSO cryoprotected CD34+ cells with addition of trehalose, an autophagy activator. (C) Representative autophagy flux level analysis of DMSO cryoprotected CD34+ cells with addition of autophagy inhibitor 3-MA. Flow cytometry plots prepared in Attune™ Cytometric software (Version 5.2.2302.0).

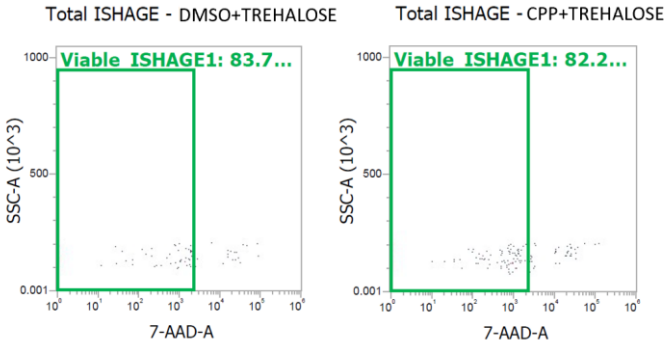
7.2. Enumeration of viable CD34+ cells cryopreserved with different CPAs and autophagy modulators using ISHAGE gating guidelines

A





B



C

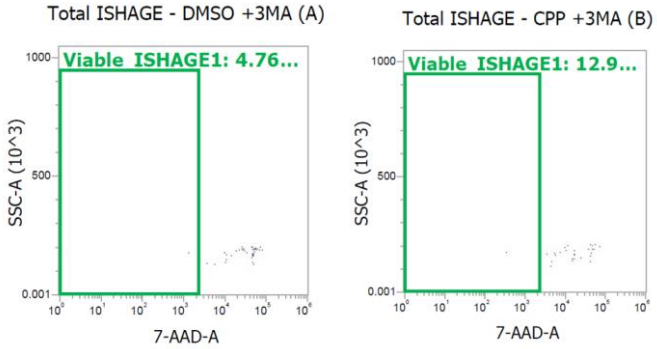


Figure 25: Representative flow cytometric analyses illustrating the impact of CPAs and autophagy modulators on CB HSPCs viability and recovery post thaw.

Expanded CD34+ cells were cryopreserved with the following condition with CPA alone autophagy modulators. After thawing, the cells were analyzed for viability, and enumeration of viable CD34+ cells (expressed in %) was performed following ISHAGE gating guidelines. (A) Representative flow cytometry analysis of DMSO cryopreserved CD34+ cells illustrating the ISHAGE strategy and CD34+ viability analysis. (B) Representative flow cytometry analysis of DMSO and CPP cryoprotected CD34+ cells with addition of trehalose. (C) Representative flow cytometry analysis of DMSO and CPP cryoprotected CD34+ cells with addition of 3-MA. Flow cytometry plots prepared in Attune™ Cytometric software (Version 5.2.2302.0).

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Parts of this research work compiled in this dissertation are published separately in the academic research journals which are listed below.

- **Publication in chapter 2:**

➢ *Publication:* Kaushal R, Jahan S, McGregor C, Pineault N. Dimethyl sulfoxide-free cryopreservation solutions for hematopoietic stem cell grafts. Cytotherapy. 2022 Mar;24(3):272-281. doi: 10.1016/j.jcyt.2021.09.002. Epub 2021 Oct 12. PMID: 34654640.

➢ *Weblink*: https://pubmed.ncbi.nlm.nih.gov/34654640/

- **Publication in chapter 5:**

➢ *Publication:* Jahan S, Kaushal R, Pasha R, Pineault N. Current and Future Perspectives for the Cryopreservation of Cord Blood Stem Cells. Transfus Med Rev. 2021 Apr;35(2):95-102. doi: 10.1016/j.tmrv.2021.01.003. Epub 2021 Jan 30. PMID: 33640254.

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