DELTA OPIOID PEPTIDE DOES NOT EXTEND VIABILITY OF REFRIGERATED HEMATOPOIETIC STEM CELLS

By

Brian Joseph Deck

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Clinical Laboratory Sciences--Master of Science

2018

ABSTRACT

DELTA OPIOID PEPTIDE DOES NOT EXTEND
VIABILITY OF REFRIGERATED
HEMATOPOIETIC STEM CELLS

By

Brian Joseph Deck

Delta opioid peptides like [D-Ala^2, D-Leu^5] Encephalin (DADLE) have been shown to improve the survivability of tissue during times of stress, like tissue transplantation or induced cardiac ischemia. The mechanism, while not well understood, has been revealed as a possible way to improve the tissue transplant process by extending the life and transport of tissues once removed from donors. The manner in which these opioid peptides effect individual cell types such as hematopoietic stem cells, is not well documented. By introducing DADLE into collections of these specific types of cells, we investigated if a similar effect took place in stem cells. Flow cytometry using CD34 enumeration and 7-AAD viability staining was used to measure DADLE activity compared to control cell treatment. Three separate experiments were performed with graded doses of DADLE and results showed there was no difference in viability between the control treated cells and the cells treated with DADLE. The results indicate that at the concentrations used, no such effect was noted and therefore further testing needs to be done to determine what factors prevented the cells from reaching a hibernation-like state.

ACKNOWLEDGMENTS

I would like to thank my thesis advisor Dr. Kathleen Hoag of the Biomedical Laboratory Diagnostics Program at Michigan State University. She consistently allowed this paper to be my own work but steered me in the right direction whenever she thought I needed it. I would also like to thank Dr. Peter Oeltgen and Dr. Antonio Torloni for their guidance and expertise in the field of Stem Cells and DADLE.

I would like to thank the American Society for Clinical Laboratory Science (ASCLS) and the Banner Foundation for their financial and facilities support with this project.

Finally, I must express my very profound gratitude to my family for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
BM	Bone Marrow
BMDACC	Banner MD Anderson Cancer Center
CD34/45	Cluster of Differentiation 34/45
CI	Confidence Interval
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DADLE	Delta Opioid Peptide [D-Ala^2, D-Leu^5] Encephalin
DFd	Degrees of Freedom Denominator
DFn	Degrees of Freedom Numerator
DMSO	Dimethyl Sulphoxide
F	Distribution of the Ratio of Two Estimates of Variance.
G-CSF	Granulocyte Colony-Stimulating Factor
HIT	Hibernation Induction Trigger
HSA	Human Serum Albumin 5%
HSC	Hematopoietic Stem Cells
IL	Interleukin
LN	Liquid Nitrogen
P	Calculated Probability
PBSC	Peripheral Blood Stem Cells

PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
RARα	Retinoic Acid Receptor Alpha
RBC	Red Blood Cells
RXR	Retinoid X Receptor
SCF	Stem Cell Factor
TNC	Total Nucleated Cells
TRα	Thyroid Hormone Receptor Alpha

INTRODUCTION

The myeloid and lymphoid lineages of cells that encompass all immune cells found in the body start with the same progenitor cell line, the hematopoietic stem cells (HSC). These stem cells originate in the red bone marrow of flat bones in adults and as they mature they get released in small amounts into the peripheral blood. Once released, HSCs circulate for a few hours in the blood stream, then return and bind to targeted hematopoietic tissue where they bind using chemokine ligand and receptor interaction (CXCR4 and CXCL12). There they will undergo multiplication and differentiation under the influence of specific cytokines in the milieu and differentiate into Red Blood Cells (RBC), White Blood Cells (WBC, including T cells, B cells, natural killer cells, monocytes and granulocytes) and megakaryocytes which will then break-up into platelets. The ability to differentiate into multiple cell types makes HSCs clinically important (1). HSCs are used in the treatment of various cancers like multiple myeloma and lymphoma, blood disorders such as aplastic anemia and myelodysplastic syndrome, and in regenerative medicine (2-4).

There are two processes that are commonly used to collect HSCs for clinical use: apheresis, which is a method of collecting hematopoietic stem cells from the peripheral blood, and bone marrow harvesting, where the cells are collected directly from the bone marrow via needle aspiration. Either collection process yields sufficient primordial cells for the desired purpose. Peripheral Blood Stem Cell (PBSC) collections and Bone Marrow (BM) harvests contain, in addition to the desired stem cells, a small quantity of platelets, polymorphonuclear leukocytes, red blood cells, as well as cellular debris. Due to the short shelf-life of a freshly

collected stem cell collection, PBSC and BM cells are usually cryopreserved following collection by control rate freezing and preserved in vapor phase or liquid phase nitrogen for later use. At 24 hours, unpreserved cells start undergoing necrosis, a breakdown of the cellular membrane, in a predictable manner and the percentage of non-viable cells can show a linear increase over time, depending on the number of cells in the storage bag and temperature of storage (5). Therefore, either PBSC or BM cells need to be cryopreserved or infused within 24 to 48 hours following collection (6).

The cryopreservation process for HSC has been well described in the literature (7,8) and involves dilution of the product and addition of cryoprotectants such as dimethyl sulphoxide (DMSO) and glycerol (8,9). These solutions protect the collected stem cells during the process of cryopreservation by balancing the osmotic pressure in the cell with the extracellular fluid and allow for simultaneous freezing of intracellular cytoplasm and organelles, nucleoplasm and chromosomes as well as nuclear and cellular membranes, therefore avoiding cell rupture. DMSO is cytotoxic in its liquid form; therefore, controlled rate freezing is started immediately after the addition of DMSO to limit its toxicity. During the cryopreservation process, there is a signature peak between negative 5°C to negative 12°C that indicates a “latent heat of fusion” (7). This marks the release of energy (in the form of heat) as the product moves completely from a liquid state to a solid state. Once the heat of fusion has occurred, the rate of freezing is accelerated until the product reaches negative 150°C. After the freezing protocol is complete, the product is immediately transferred to a large liquid nitrogen (LN) storage tank and kept in the vapor phase. This process is commonly used, and it has been shown that the viability of cells remains constant for many years (3,10). When required, one or more of the storage bags

containing stem cells are thawed using a 37°C water bath then infused into the recipient. If the cells are not for immediate use, washing will remove the DMSO and allow the cells to be used within hours.

Although other methods are used, controlled rated cryopreservation with DMSO is the industry standard for preserving HSCs. This process is not without its challenges though. For most stem cell collections, the collected cells must undergo either minimal or more than minimal manipulation. These types of manipulation include volume reduction, plasma or red cell reduction, cell isolation, transfer into different bags, and sometimes product washing. Each one of these manipulation methods can have a dramatic effect on the cells, resulting in cell death and/or decreased viability. The cryopreservation process itself can cause a 37% (±25%) drop in the total nucleated cell (TNC) viability due to the variation in freezing standards (8, 9) and DMSO toxicity (3,11). While these drops in viability can affect the efficacy of the stem cell product, they are widely accepted due to the lack of a better process. Is there a better way to preserve stem cells without the viability being affected?

The goal of cryopreservation is to slow down or stop cellular metabolic activity. Nature has a similar process of slowing down metabolic pathways. Hibernation is a state of inactivity and extreme metabolic depression (12). During hibernation, an animal's body temperature decreases, the heart rate drops, and all metabolic activity slows down. This enables the hibernating animal to conserve energy during times when food sources are lacking. Hibernation in mammals is triggered by a decrease daylight (shorter days) that precedes winter. Researchers have found a protein factor in the plasma of winter hibernating animals that helps to induce hibernation and slow down the metabolic rate (12). This 88-kD peptide, hibernation

induction trigger (HIT) factor, has been shown to initiate hibernation in multiple studies, even in species that don't normally hibernate (13) or in hibernation animals during the summer months (14, 15). It was this activity that lead to testing HIT in the field of organ transplantation.

Studies have shown that when HIT is introduced into the blood stream of a donor organ, the survival period of the organs once removed increased dramatically (16,17). HIT has been used in research with extracted lungs, hearts, and livers to show its effectiveness in increasing the shelf life of each organ once removed from the body (17,18). It was shown that this increase in organ viability over time was due to a drop in the metabolic rate of the cells composing the organs, slowing the process of cell death. Using protein electrophoresis, Oltgen et al found HIT to be a protein that is tightly bound to albumin (15) and subsequently sequenced. It was determined to be a delta opioid.

There is a delta opioid compound that mimics the abilities of HIT (11,17). Delta opioid peptide [D-Ala^2, D-Leu^5] encephalin (DADLE) is a synthetic opioid that has been tested side-by-side with HIT to show a correlation in the effects (13, 17-20). Studies have shown that DADLE interacts with the μ opioid receptors on organ cells and with δ opioid receptors in ischemic studies (20,21,22). DADLE has also been shown to have an antiapoptic effect on individual cell lines like pheochromocytoma cells (PC12), SH-SY5Y, and primary cortical neuron cells (23,24). In the presence of other cells where opioid receptors are not present, DADLE is able to permeate the cell membrane via the use of Na^+-coupled membrane transporters (25, 26). The effects of DADLE have been shown to be reversible, similar in nature to an animal coming out of the hibernation state (27).

MATERIALS AND METHODS

Peripheral blood HSC's were obtained through the Stem Cell Therapy Lab at Banner MD Anderson Cancer Center (BMDACC), an FDA licensed stem cell lab. These cells were previously cryopreserved stem cells that had been released for research purposes. The donors for these cells were consented through the Stem Cell Program at BMDACC. As these products were released for research by the Medical Director, no pertinent patient information will be shared and no human subject consent for this experiment was necessary. Once the product was released by the Medical Director, it was labeled according to research standards within the BMDACC Stem Cell Program.

To obtain statistically significant data for proper analysis and show reproducibility, the experiment was run 3 separate times (phase 1, phase 2, and phase 3). For each run, the CD34+ stem cell products were thawed and washed according to applicable standard operating procedures therefore removing possible damage by liquid DMSO used for cryopreservation. This process was performed on the COBE 2991® cell washer/processor (Terumo BCT). After the cells were washed, to allow for sufficient testing volume and to best mimic the current collection process, the cells were brought up to a total of 960 mL using the remaining cell volume mixed with equal parts of sterile 0.9% Sodium Chloride Injection USP (saline) (Baxter, catalog #2B1309) and 4% Anticoagulant Citrate Dextrose (ACD-A) (Fenwal, catalog #487891X) to create an isotonic solution for the cells suspension. The ACD-A, a mixture of dextrose (monohydrate), sodium citrate (dihydrate), and citric acid (anhydrous), creates a buffered solution for the cells. Once the volume was brought up to the required amount (480 mL) the

cell solution was split into 6 different bags, each containing an average of 39 x 10^6 CD34+ cells (range of 23 x 10^6 – 53 x 10^6) (Figure 1).

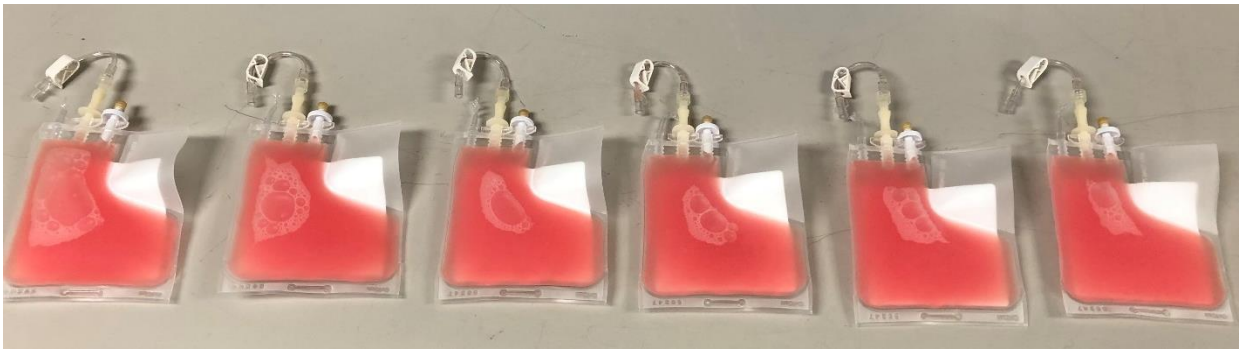

Figure 1. DADLE dosed stem cell product bags, each containing a different dose of DADLE or no DADLE (control).

Since no data exists pertaining to the effects of DADLE on stem cells, a range of concentrations was used to determine if there was an ideal dose effect (with the least amount of toxicity). Studies have shown that DADLE can influence metabolic activity in tissues and other cell lines between concentrations of 100 pM and 1 µM without toxic effects. At 1 µM and beyond, DADLE can become cytotoxic to the system (24). DADLE (Sigma-Aldrich, catalog # 94825-57-7) is a hydrolysis-resistant compound (25,26) which allows it to be stable in solvents like water or saline. For this experiment it was suspended in 0.9% saline and 4% ACD-A to protect the stem cells from inadvertent damage. The control dose was 0 M, which showed the typical response of stem cells over time. To create the correct doses, serial dilutions were performed starting with the largest dose (1 µM) and further diluted until the final dose was reached (100 pM). The molecular weight of DADLE was used to calculate how many grams were needed to create a solution that was 10 µM. This was the starting dose for creating serial dilutions. The other 5 doses used were: 100 pM, 1 nM, 10 nM, 100 nM, and 1 µM. Each of the

bags were labeled with the corresponding dose of DADLE to be added as well as the experiment phase and start date.

Once the cell suspension was split and the doses of DADLE added, each bag was thoroughly mixed and stored at 2-4°C. To test for the effects (if any) of DADLE, samples were taken from each bag every 8 hours to measure total nucleated cell (TNC) count, 7-AAD viability, and CD34/45 enumeration. Bags were mixed gently before sampling to ensure a homogenous mixture (Figure 2). Samples were taken every 8 hours over a period of 88 hours (between 3.5-4 days) using aseptic technique (sterile needles and syringes for each sample, samples taken under the biosafety hood, port wiped down with alcohol swap before sampling). This allowed the viability counts to be tested for 2 full days longer than the suggested/recommended shelf-life of 48 hours currently used for collected stem cells.

Figure 2. Cell product bags before mixing. The white layer seen at the top of the bag represents a layer of saline and possibly ACD-A (both of which have a lower specific gravity than plasma).

All instruments required for testing were located in the Cell Therapy Lab at BMDACC. For the TNC testing, a Sysmex XS1000i® (Sysmex) was used, which is specifically calibrated for stem cell populations and has a linearity of 0-440 thousand white blood cells per µL. Background check and quality control analysis were run daily prior to testing to ensure instrument was performing within specifications. The system requires at least 20 µL of sample to be run and the samples were run both neat (undiluted) and diluted to a ratio of 1:10.

CD34/CD45 enumeration was performed using the FACS Canto II® flow cytometer (BD Biosciences), also calibrated and used specifically for stem cell populations. CD34 and CD45 are antigens found on immature hematopoietic precursor cells in bone marrow and blood, including unipotent and pluripotent progenitor cells. CD45 antigens are expressed on all hematopoietic cells except for RBCs. CD34 antigens are a specific subset of the CD45 cells, the hematopoietic stem cells. CD34 positive cells will normally make up about 0.1% of all CD45 cells.

The antibodies that were used for CD34 and CD45 testing were each composed of mouse IgG_1 heavy chains and kappa light chains. These antibodies are combined with the fluorochromes FITC (CD45) and PE (CD34) in phosphate buffered solution containing bovine serum albumin and 0.1% sodium azide. Antibodies and 7-AAD dye were purchased from BD Diagnostics. The blue laser was utilized for FITC and PE detection.

The flow cytometer measures both the presence of the antigens and the viability of the cells. Prior to testing on the flow cytometer, the samples were stained per the BD Bioscience protocol. This protocol includes:

1. Pipette 20 µL of CD34/CD45 reagent into a test tube.

2. Pipette 20 µL of 7-AAD viability dye into the same test tube.
3. Pipette 100 µL from the sampling tube that was pulled from the corresponding dose bag and add it to the test tube.
4. Vortex gently then incubate at room temperature for 30 min, in the dark.
5. Pipette 2 mL of lysing solution to the test tube after 30 min incubation.
6. Vortex gently then incubate at room temperature for 10 min, in the dark.
7. Run sample on the flow cytometer after 10 min incubation.

The test results were then compiled, and the data was analyzed using GraphPad Prizm®.

RESULTS

To test whether DADLE has a hibernating effect on peripherally collected hematopoietic stem cells, 7-AAD viability testing was run on samples from 6 different bags containing different doses of DADLE (control bag had no DADLE added). Each of the bags were tested every 8 hours for a total of 88 hours. Figure 3 shows the regression line for each of the doses, including the 95% confidence interval for each. For this data F = 0.4052, DFn = 5, DFd = 60, P=0.8434. If the overall slopes were identical, there is an 84.34% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are not significant. Using the Tukeys multiple comparisons test (Table 1), each of the doses were compared to each other to determine p value and significance in difference (using GraphPad Prizm). There was no significant difference between the control dose and the other 5 doses.

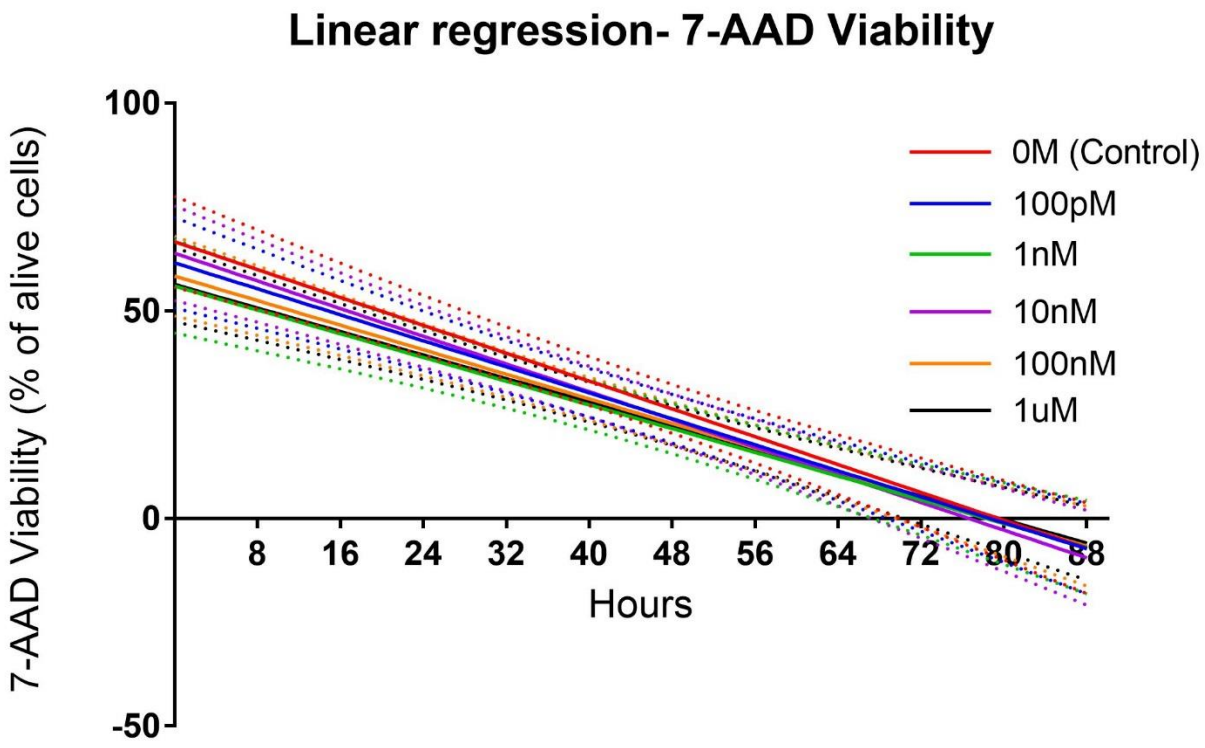

Figure 3. 7-AAD viability testing for all doses up to 88 hours. 7-AAD is a fluorescent chemical compound that stains the internal parts of cells if the cell membrane is disrupted (due to necrosis or cell death). The viability that is measured depicts the number of live cells when compared to the total number of cells (live and dead). Viability at the start of experiment was above 50%. The pooled slope equals -0.7694.

Table 1. 7-AAD viability Tukeys multiple comparisons test using all 6 doses. Comparisons include means, 95% CI, and adjusted P values.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
0M (Control) Viability vs. 100pM Viability	2.614	-3.054 to 8.282	No	0.7667
0M (Control) Viability vs. 1nM Viability	5.251	-0.4172 to 10.92	No	0.0865
0M (Control) Viability vs. 10nM Viability	2.546	-3.122 to 8.214	No	0.786
0M (Control) Viability vs. 100nM Viability	3.898	-1.77 to 9.566	No	0.3551
0M (Control) Viability vs. 1uM Viability	4.553	-1.115 to 10.22	No	0.1927
100pM Viability vs. 1nM Viability	2.637	-3.031 to 8.305	No	0.7601
100pM Viability vs. 10nM Viability	-0.06806	-5.736 to 5.6	No	>0.9999
100pM Viability vs. 100nM Viability	1.284	-4.384 to 6.952	No	0.9865
100pM Viability vs. 1uM Viability	1.939	-3.729 to 7.607	No	0.9211
1nM Viability vs. 10nM Viability	-2.705	-8.373 to 2.963	No	0.7399
1nM Viability vs. 100nM Viability	-1.353	-7.021 to 4.316	No	0.9829
1nM Viability vs. 1uM Viability	-0.6975	-6.366 to 4.971	No	0.9992
10nM Viability vs. 100nM Viability	1.352	-4.316 to 7.02	No	0.9829
10nM Viability vs. 1uM Viability	2.007	-3.661 to 7.675	No	0.9096
100nM Viability vs. 1uM Viability	0.655	-5.013 to 6.323	No	0.9994

To corroborate the viability data, linear regressions and Tukeys multiple comparisons were also created using the total CD34 (Figure 4 and Table 2). For this data F = 0.5023, DFn = 5, DFd = 60, P=0.7733. If the overall slopes were identical, there is a 77.33% chance of randomly choosing data points with slopes this different. You can conclude that the differences between the slopes are not significant. Since the slopes are not significantly different, it is possible to calculate one slope for all the data. There is one comparison in the CD34 Tukeys table that did show a significant difference. The mean difference between the control dose and the 1µM dose had a P value of 0.02, making it significant enough to stand out.

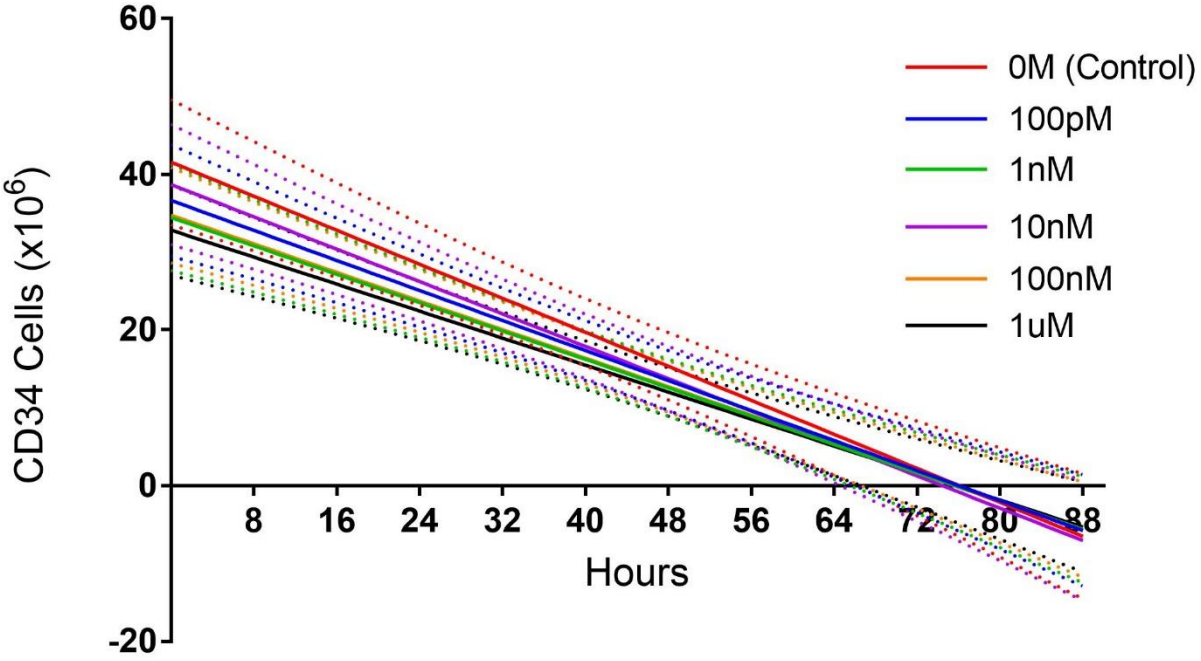

Figure 4. Total count of CD34 cells performed on the flow cytometer per dose over the course of 88 hours. CD34 cells account for 0.01% of the total CD45 cell population (which includes all nucleated white cells). The pooled slope equals -0.4827.

Table 2. CD34 Tukeys multiple comparisons test using all 6 doses. Comparisons include means, 95% CI, and adjusted P values.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
0M (Control) CD34 vs. 100pM CD34	2.083	-1.303 to 5.469	No	0.4837
0M (Control) CD34 vs. 1nM CD34	3.115	-0.2706 to 6.501	No	0.0904
0M (Control) CD34 vs. 10nM CD34	1.698	-1.688 to 5.084	No	0.6973
0M (Control) CD34 vs. 100nM CD34	2.972	-0.4137 to 6.358	No	0.1208
0M (Control) CD34 vs. 1uM CD34	3.762	0.3763 to 7.148	Yes	0.02
100pM CD34 vs. 1nM CD34	1.032	-2.354 to 4.418	No	0.9507
100pM CD34 vs. 10nM CD34	-0.3853	-3.771 to 3.001	No	0.9995
100pM CD34 vs. 100nM CD34	0.8889	-2.497 to 4.275	No	0.9739
100pM CD34 vs. 1uM CD34	1.679	-1.707 to 5.065	No	0.7074
1nM CD34 vs. 10nM CD34	-1.417	-4.803 to 1.969	No	0.8319
1nM CD34 vs. 100nM CD34	-0.1431	-3.529 to 3.243	No	>0.9999
1nM CD34 vs. 1uM CD34	0.6469	-2.739 to 4.033	No	0.9938
10nM CD34 vs. 100nM CD34	1.274	-2.112 to 4.66	No	0.8859
10nM CD34 vs. 1uM CD34	2.064	-1.322 to 5.45	No	0.4942
100nM CD34 vs. 1uM CD34	0.79	-2.596 to 4.176	No	0.9845

Total CD45 data also corroborates the viability data (Figure 5 and Table 3). For this data F = 0.3294, DFn = 5, DFd = 60, P=0.8933. If the overall slopes were identical, there is an 89.33% chance of randomly choosing data points with slopes this different. You can conclude that for the total CD45, the differences between the slopes are not significant.

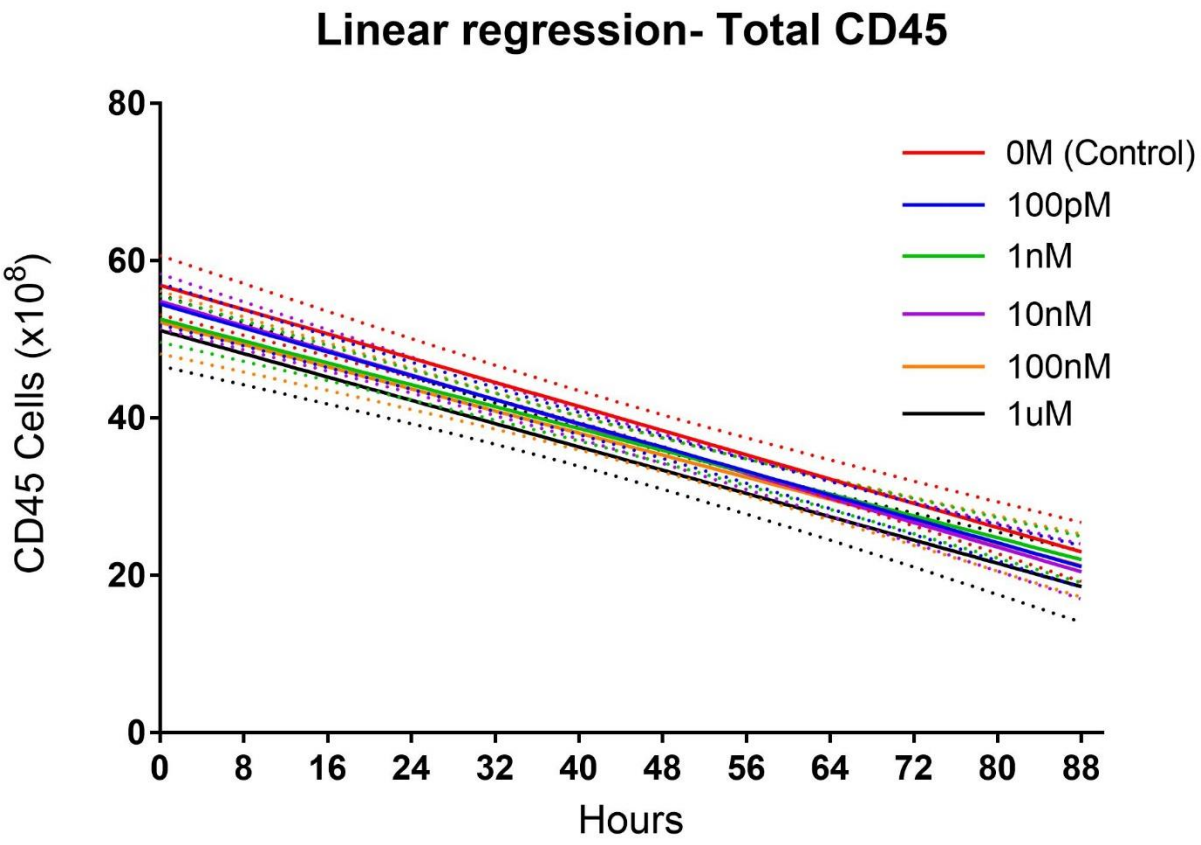

Figure 5. Total count of CD45 cells (which include the subset of CD34 cells) done on the flow cytometer per dose over the course of 88 hours. The pooled slope equals -37.08.

Table 3. CD45 Tukeys multiple comparisons test using all 6 doses. Comparisons include means, 95% CI, and adjusted P values.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
0M (Control) CD45 vs. 100pM CD45	2.133	-13.34 to 17.61	No	0.9987
0M (Control) CD45 vs. 1nM CD45	2.649	-12.83 to 18.12	No	0.9963
0M (Control) CD45 vs. 10nM CD45	2.279	-13.2 to 17.75	No	0.9982
0M (Control) CD45 vs. 100nM CD45	3.239	-12.24 to 18.71	No	0.9906
0M (Control) CD45 vs. 1uM CD45	5.075	-10.4 to 20.55	No	0.9333
100pM CD45 vs. 1nM CD45	0.5152	-14.96 to 15.99	No	>0.9999
100pM CD45 vs. 10nM CD45	0.1452	-15.33 to 15.62	No	>0.9999
100pM CD45 vs. 100nM CD45	1.105	-14.37 to 16.58	No	>0.9999
100pM CD45 vs. 1uM CD45	2.942	-12.53 to 18.42	No	0.994
1nM CD45 vs. 10nM CD45	-0.37	-15.84 to 15.1	No	>0.9999
1nM CD45 vs. 100nM CD45	0.59	-14.88 to 16.06	No	>0.9999
1nM CD45 vs. 1uM CD45	2.426	-13.05 to 17.9	No	0.9976
10nM CD45 vs. 100nM CD45	0.96	-14.51 to 16.43	No	>0.9999
10nM CD45 vs. 1uM CD45	2.796	-12.68 to 18.27	No	0.9952
100nM CD45 vs. 1uM CD45	1.836	-13.64 to 17.31	No	0.9994

DISCUSSION

Although the current process of freezing hematopoietic stem cells varies amongst transplant centers such as rate of freezing and percent concentration of DMSO, there have been no major changes to the overall process/objective for over 20 years (3). While this process gets the end users to the cryopreservation goal, it's not without risks. Testing different processes to improve the overall outcome of stem cell usage is important and necessary, even if the new processes/ideas do not work as expected. My study goal was to determine if the described effects of DADLE on tissue preservation could be extended to preserving HSC viability without cryopreservation. The findings from this study suggest that D-Ala^2, D-Leu^5 Enkephalin (DADLE) did not demonstrate a statistically significant effect on the viability or longevity of peripherally collected hematopoietic stem cells held in vitro at refrigeration temperature.

While the data did not support the hypothesis, the P values do lend themselves to a possible trend. The P value for the comparison of total CD34 between the control dose and the 1µM dose was significant (0.02). This data point highlighted something that could be a trend pertaining to how toxic the doses could have been to the cells. On further review of the Tukeys multiple comparison tables there is a decrease in P value as the control dose is compared to increasing doses of DADLE. This could mean that the doses used in this project were toxic to the cells from the start. This notion can be further suggested by looking at the linear regressions. In each of the graphs, the control dose remains higher than the other doses for the entirety of the experiments while the largest dose (1µM) remained at the bottom of the

grouping. While this suggests that the doses used may have been toxic or trending towards toxicity, more research would be needed to determine this.

Previous studies that tested the ability of DADLE to affect viability and longevity of cell systems used whole organ tissue like hearts and lungs as well as individual cell lines (16-18). These studies demonstrated that dropping the metabolic rate in the cells that compose the organs was possible, which in turn slowed the process of cell death. DADLE was infused through the organs prior to extraction/removal to ensure enough DADLE was present to have the desired effect. It was shown that DADLE interacts with μ and δ opioid receptors found on the organ tissue cells (14,20,21,22). If those receptors were not present, it was also shown that DADLE could permeate the cell membrane via Na^+- coupled membrane transporters (25,26). While these routes have been verified through research, the actual mechanism of how DADLE interacts with the cells is still uncertain and under investigation (22,23).

The current project differed from previous experiments due to the type of cells that were targeted for DADLE treatment and how those cells were exposed to the peptide. The cells utilized had already been frozen and here thawed for the study. There are multiple types of cells working in congruence that make the lung function the way that it does and DADLE could have interacted with each one of those cell types. In a study by T. Hayashi, DADLE was tested against PC12 cells and found to have antiapoptic effects between femtomolar and picomolar concentrations (24). With my project, there was only one cell type that was tested, the hematopoietic stem cells. To best test this specific cell type I used a wide range of doses that were used in the previous studies. Whether these doses are the best for hematopoietic stem cells is unknown, there were no previous studies that offered that information.

For the current study, the cells were not introduced to DADLE until after they had been collected and stored. These cells do not have the ability of being exposed to DADLE prior to apheresis/removal like the lung and heart tissue were previously, which can also impact how DADLE interacts with the cells. In previous studies, perfusion prior to organ removal and perfusion after removal/extraction were performed. Preconditioning with DADLE had a large impact on how DADLE affected the cells.

Temperature may have also been a factor in this study. The previous studies that tested DADLE in its effect on transplanted organs were done at room temperature or in at least one case using a 37°C water bath (17). The initial contact that DADLE had with the organ cells happened inside the organ donor. The current study introduced DADLE into the cell product while at 2°-4°C and then stored at those temperatures throughout the experiment. This temperature difference may have prevented DADLE from properly penetrating the stem cells and/or appreciably changing their metabolic activity. While the goals of previous studies and this current study were similar (extending the life of cells, decreasing cell metabolism) there were quite a few differences that makes it difficult to compare them.

Aside from the differences in the previous and current experiments, there were also methodological limitations to this current study. It would have been ideal and closer to the normal stem cell process if fresh cells were used rather than previously frozen cells. Obtaining fresh cells would have been more difficult due to the increase in financial need, possible adjustment in IRB approval (now you are involving live patients who need to consent for collection of cells), and timing. I was also limited in what types of testing could be performed, although the testing that was used was enough to get the data needed to test my hypothesis.

Viability and CD34/45 counts were the central tests that needed to be performed to obtain adequate information. Additional tests would have been good in backing up the data and possibly explaining what was going on at a cellular level when DADLE was introduced to the stem cells. Microscopy was used minimally to show the differences in appearance of cells after hour 88 (Figure 6).

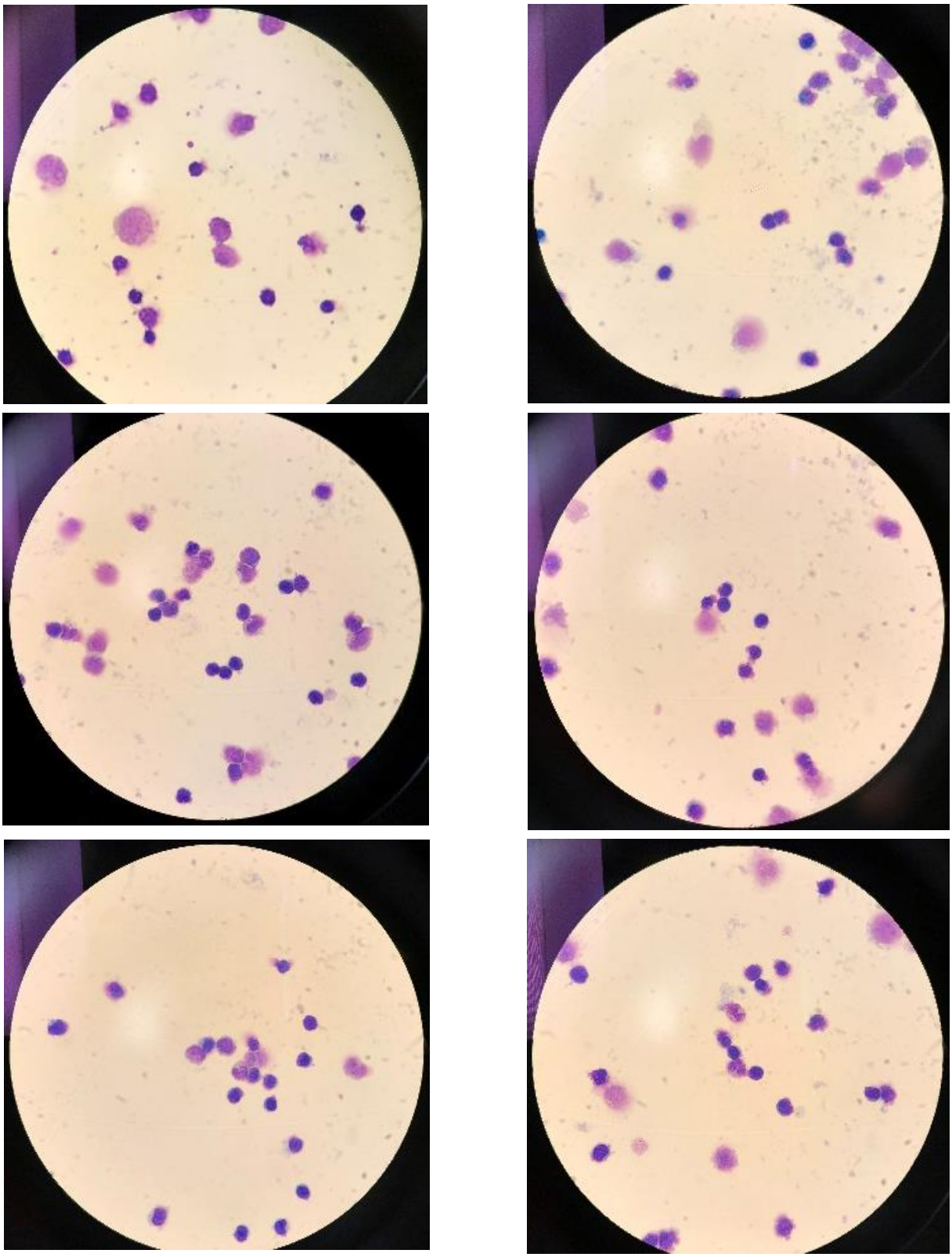

Figure 6. Stained PBSC at 88 hours. DADLE doses, starting with top-left: 0M, 100pM, 1nM,

Figure 6 (cont'd). 10nM, 100nM, and 1μM. Slides are stained with Wright's stain, a mixture of eosin (red) and methylene blue dyes.

Receptors and Dosing

There are many possibilities as to why the current experiment did not support my original hypothesis. One of the unknowns that warrants a discussion deals with cell receptors. HSCs have been shown to have receptors for interleukin-1α (IL-1α), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), and c-kit (receptor for stem cell factor [SCF]) (28). They also have various receptors (like RARα, RXR, TRα, and PPARγ) related to how the cells differentiate when exposed to the necessary triggers (29). Studies have shown that CD34+ cells have κ and μ opioid receptors (30) but it is not well known if these cells have δ receptors. The data from the current study does not fully support the presence or adherence of DADLE to μ and δ receptors but more research would be needed to find out for sure.

Another possibility as to why the data does not support the hypothesis pertains to the dosing of DADLE. In previous studies, dosing was based on the tissue being studied. This dosage was introduced into the organ tissue systems prior to removal so it was continually being exposed to DADLE over time. In the current experiment, DADLE was introduced to the HSCs at the start of each phase and remained in a steady state through the entirety of the phase instead of circulating through the solution.

Reagents and Additives

For the current investigation, reagents like DMSO, HSA, and saline were used during processing and storage of the cell products. The effects that these reagents have on DADLE are

unknown and could have played a role in the lack of reaction on the HSCs. DMSO is known to have a toxic effect on stem cells after products are thawed from cryopreservation. Therefore, there is a time limit to how long the stem cell product is usable once thawed (30 min-2 hours). To extend the life of the stem cells, the cells were washed so that the DMSO could be removed from the cell product solution. The process of washing cells can also have an effect on the cells, but that affect is still more beneficial than having the cells exposed to DMSO for an extended amount of time. Could the addition of glycerol to the cell mixture provide more of the needed environment for the cells to be able to absorb DADLE?

While stem cells can be stored and remain healthy in HSA and saline, it may be more beneficial for the cells to be suspended in a solution like plasma. This would more closely mimic the environment that they normally thrive in. While more ideal, this would add some additional complexities to the experiment due to the need of plasma that is either a match to the cells being tested (the wrong ABO type could potentially react with any residual RBCs in the product) or plasma that has been depleted of all antibodies.

Are there other additives that I could have used to aid in the increased permeability of the stem cells, allowing DADLE to have more of an effect? Stem cell factor is a cytokine that helps HSCs survive longer *in vitro* and helps in the maintenance and self-renewal of HSCs *in vivo*. Once concern in using this as an additional additive is its propensity to activate mast cells (due to mast cells having the same receptors). Another possible additive, IL-7 (Interleukin 7), is a growth factor that is normally secreted by stromal cells in the bone marrow and thymus. This cytokine stimulates HSCs to differentiate into lymphoid progenitor cells.

Further Research

Studies investigating the effects of DADLE on organs and tissue are numerous and offer great insight into improving the transplant process for patients. Studies using stem cells though are virtually nonexistent and more research is needed in this field. Original studies done by Dr. Oltgen used HIT found in the plasma fractions of woodchucks, not DADLE. Do stem cells have other receptors besides mu opioid receptors, that are necessary for peptides like DADLE? What methodology will provide the best data and outcomes for testing DADLE with stem cells? What doses of DADLE would show an effect on stem cell viability? What is the best temperature to allow cellular hibernation response to DADLE? Would it be more beneficial to use one of the 5 isomers of HIT instead of DADLE? Could DADLE have a different isomer configuration that may affect the *in vivo* function? These are some of the questions that would need to be answered to fully understand the effects, if any, DADLE could have on extending the life span of *in vitro* stored hematopoietic stem cells.

CONCLUSION

Stem cell transplants and cellular therapies are becoming more prevalent in the treatment of various types of cancers. The current procedures for storing patient stem cells require the use of specialized equipment and reagents to freeze the cells and store long term. These procedures are the industry standard, but they do have their limitations and possible negative impact when it comes to cell therapy products. This study focused on alternative methods to preserving stem cell products in hopes of avoiding the negative effects of freezing and thawing. Although the data from this study did not support the original hypothesis, it does show that more investigation into alternative storage methods is needed. The ability to store HSCs for long periods of time without the need to freeze and thaw them would greatly improve the efficacy of the cell product and the efficiency of the process.

BIBLIOGRAPHY

BIBLIOGRAPHY

1) Lauterboeck, L., N. Hofmann, T. Mueller, B. Glasmacher, Active control of the nucleation temperature enhances freezing survival of multipotent mesenchymal stromal cells. *Cryobiology* **71**, 384-390 (2015).

2) Li, X. L. *et al.*, Hematopoietic stem cells: cancer involvement and myeloid leukemia. *Eur Rev Med Pharmacol Sci* **19**, 1829-1836 (2015).

3) Berz, D., E. M. McCormack, E. S. Winer, G. A. Colvin, P. J. Quesenberry, Cryopreservation of hematopoietic stem cells. *Am J Hematol* **82**, 463-472 (2007).

4) Dauber, K. *et al.*, Enumeration of viable CD34(+) cells by flow cytometry in blood, bone marrow and cord blood: results of a study of the novel BD™ stem cell enumeration kit. *Cytotherapy* **13**, 449-458 (2011).

5) Antonenas, V. *et al*., Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport. *Cytotherapy* **8**, 158-165 (2006).

6) Pamphilon, D., A. Mijovic, Storage of hemopoietic stem cells. *Asian J Transfus Sci* **1**, 71-76 (2007).

7) Morris, J., Asymptote Guide to Cryopreservation, second ed., Asymptote Ltd. (2007).

8) Humpe, A. *et al.*, Comparison of CD34+ cell numbers and colony growth before and after cryopreservation of peripheral blood progenitor and stem cell harvests: influence of prior chemotherapy. *Transfusion* **37**, 1050-1057 (1997).

9) Winter, J. M. *et al*., Long-term effects of cryopreservation on clinically prepared hematopoietic progenitor cell products. Cytotherapy 16, 965-975 (2014).

10) Galmés, A. *et al.*, Long-term storage at -80 degrees C of hematopoietic progenitor cells with 5-percent dimethyl sulfoxide as the sole cryoprotectant. *Transfusion* **39**, 70-73 (1999).

11) Windrum, P. *et al.*, Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. *Bone Marrow Transplant* **36**, 601-603 (2005).

12) Su, T. P., Delta opioid peptide[D- Ala(2),D-Leu(5)]enkephalin promotes cell survival. *J Biomed Sci* **7**, 195-199 (2000).

13) Borlongan, C. V., Y. Wang, T. P. Su, Delta opioid peptide (D-Ala 2, D-Leu 5) enkephalin: linking hibernation and neuroprotection. *Front Biosci* **9**, 3392-3398 (2004).

14) Sonneborn, J. S., H. Gottsch, E. Cubin, P. Oeltgen, P. Thomas, Alternative strategy for stress tolerance: opioids. *J Gerontol A Biol Sci Med Sci* **59**, 433-440 (2004).

15) Horton, N. D. *et al.*, Isolation and partial characterization of an opioid-like 88 kDa hibernation-related protein. *Comp Biochem Physiol B Biochem Mol Biol* **119**, 787-805 (1998).

16) Chien, S. *et al.*, Two-day preservation of major organs with autoperfusion multiorgan preparation and hibernation induction trigger. A preliminary report. *J Thorac Cardiovasc Surg* **102**, 224-234 (1991).

17) Oeltgen, P. R., N. D. Horton, S. F. Bolling, T. P. Su, Extended lung preservation with the use of hibernation trigger factors. *Ann Thorac Surg* **61**, 1488-1493 (1996).

18) Chien, S., P. R. Oeltgen, J. N. Diana, R. K. Salley, T. P. Su, Extension of tissue survival time in multiorgan block preparation with a delta opioid DADLE ([D-Ala2, D-Leu5]-enkephalin). *J Thorac Cardiovasc Surg* **107**, 964-967 (1994).

19) Borlongan, C. V., T. Hayashi, P. R. Oeltgen, T. P. Su, Y. Wang, Hibernation-like state induced by an opioid peptide protects against experimental stroke. *BMC Biol* **7**, 31 (2009).

20) Oeltgen, P. R., M. Govindaswami, D. B. Witzke, 24-hour pretreatment with delta opioid enhances survival from hemorrhagic shock. *Acad Emerg Med* **13**, 127-133 (2006).

21) McBride, S. M., J. Smith-Sonneborn, P. Oeltgen, F. W. Flynn, delta2 opioid receptor agonist facilitates mean arterial pressure recovery after hemorrhage in conscious rats. *Shock* **23**, 264-268 (2005).

22) Bolling, S. F. *et al.*, Opioids confer myocardial tolerance to ischemia: interaction of delta opioid agonists and antagonists. *J Thorac Cardiovasc Surg* **122**, 476-481 (2001).

23) Tian, J. *et al.*, [D-Ala2, D-Leu5] encephalin (DADLE) reversibly inhibits cellular transcription in neurons without causing cell injury. *Brain Res* **1565**, 1-7 (2014).

24) Hayashi, T., L. I. Tsao, T. P. Su, Antiapoptotic and cytotoxic properties of delta opioid peptide [D-Ala(2),D-Leu(5)]enkephalin in PC12 cells. *Synapse* **43**, 86-94 (2002).

25) Ananth, S. *et al.*, Transport of the synthetic opioid peptide DADLE ([D-Ala2,D-Leu5]-enkephalin) in neuronal cells. *J Pharm Sci* **101**, 154-163 (2012).

26) Chothe, P., N. Singh, V. Ganapathy, Evidence for two different broad-specificity oligopeptide transporters in intestinal cell line Caco-2 and colonic cell line CCD841. *Am J Physiol Cell Physiol* **300**, C1260-1269 (2011).

27) Vecchio, L. *et al*., DADLE induces a reversible hibernation-like state in HeLa cells. *Histochem Cell Biol* **125**, 193-201 (2006)

28) McKinstry, W., C. Li, J. Rasko, N. Nicola, G. Johnson, D. Metcalf, Cytokine Receptor Expression on Hematopoietic Stem and Progenitor Cells. *Blood* **89**, 65-71 (1997).

29) Chute, J., J. Ross, D. McDonnell, Minireview: Nuclear Receptors, Hematopoiesis, and Stem Cells. *Mol Endocrinol.* **24**(1), 1-10 (2010).

30) Steidl, U. *et al.,* Primary human CD34 hematopoietic stem and progenitor cells express functionally active receptors of neuromediators. *Blood*. **104**(1), 81-88.