

**Cryopreservation of Peripheral Blood Progenitor Cells By Uncontrolled  
Rate Freezing at -95 degrees C Using Cryoprotectant Containing a Final  
Concentration of 5 Percent Dimethyl Sulfoxide**

## **ABSTRACT**

**BACKGROUND:** The increased use of HPC transplantation has led to a need for greater efficiency in cryopreservation. This study evaluates the use of cryoprotectant consisting of a final DMSO concentration of five percent and a -95C uncontrolled rate freezing method.

**STUDY DESIGN AND METHODS:** Cryopreservation of 52 units was performed using cryoprotectant containing a final concentration of five percent DMSO. WBC counts, CD34 analysis, 7-AAD, cultures, and trypan viability testing were performed. Viabilities and engraftment rates from 15 transplants were compared to patients transplanted with units cryopreserved using cryoprotectant with a final DMSO concentration of 10 percent.

**RESULTS:** Units cryopreserved using five percent DMSO concentration showed an average viability of 93.86 percent, compared to 88.87 percent for units cryopreserved using a 10 percent DMSO concentration. Engraftment rates for 10 percent DMSO concentration were 10.4 days for ANC and 14.6 days for platelets, compared to 11.67 days for ANC and 14.07 days for platelets when using a five percent DMSO concentration.

**CONCLUSION:** This study shows HPC products cryopreserved at -95C using cryoprotectant consisting of a final concentration of five percent DMSO provides unaffected viability and engraftment rates when compared to units cryopreserved with of a final DMSO concentration of 10%.

## INTRODUCTION

The use of hematopoietic progenitor cells (HPC) collected by apheresis for transplantation as a treatment for hematological diseases has increased in recent years<sup>1</sup>. This increased demand requires added efficiencies for product collection, cryopreservation, storage, and infusion. There has also been a parallel trend to decrease Dimethyl Sulfoxide (DMSO) concentrations in cryoprotectant solutions to help prevent transplant patient toxicity<sup>2</sup>. Current methods of HPC cryopreservation commonly use a controlled-rate freezing method<sup>3-8</sup>. This method involves the use of a controlled rate freezer which is programmed specifically to allow for a slow reduction in temperature (approximately 1 degree C/minute) within the freezing chamber<sup>3-8</sup>. Controlled cooling continues until the product reaches a specified temperature, usually <-80 degrees C. Upon reaching <-80 degrees C, the product is moved into permanent storage in a liquid or vapor phase liquid nitrogen freezer. The process of controlled rate freezing can be very time-consuming and involves expensive equipment<sup>8</sup>. A simpler, and less common, method of cryopreservation involves uncontrolled-rate freezing of the HPC products by placing the product directly into a -80 degrees C freezer. The product remains there, undisturbed, and is later stored in a -80 degrees C mechanical freezer or a liquid or vapor phase nitrogen freezer<sup>3-10</sup>.

Cryopreservation of HPC products requires the use of a cryoprotective agent during the cryopreservation process. This cryoprotective agent protects the cells during the intense freezing process. Dimethyl sulfoxide (DMSO) is the most common intracellular cryoprotectant used today. The final concentration of DMSO found in cryopreserved products using an uncontrolled-rate freezing method ranges from 5-10 percent and 2.2-10 percent for controlled rate freezing

methods. The DMSO is used along with extracellular protective agents such as human serum albumin (HSA), plasma, serum, and solutions of hydroxyethyl starch (HES)<sup>3,11,12</sup>. A reduction in the amount of DMSO used in cryopreservation would be beneficial to transplant patients as it would decrease the risk of DMSO toxicity<sup>2</sup>.

This study presents the results of hematopoietic reconstitution in 15 patients using peripheral blood hematopoietic progenitor cells cryopreserved using uncontrolled rate freezing at -95 degrees C. The cryoprotectant used consists of 25 percent HSA, six percent Hetastarch in Lactated Ringers solution, and five percent DMSO (final product concentration). The quality of the product thus prepared is evaluated in terms of cell viability and the course of engraftment (hematopoietic reconstitution). Finally, a comparison of viability and engraftment rates with a previous cryoprotectant, consisting of a final concentration of 10 percent DMSO, six percent HES, albumin, and Plasma-Lyte A, will be performed.

## **MATERIALS AND METHODS**

From March 2008 through December 2008, 15 patients underwent peripheral blood HPC collection. These collections were cryopreserved using a solution consisting of 25 percent HSA, six percent Hetastarch in Lactated Ringers solution, and five percent DMSO (final product concentration). Once cryoprotectant was added to the product, the products were frozen at -95 degrees C using uncontrolled rate freezing. Table 1 summarizes the patients' characteristics/diseases.

For each individual collection, cryoprotectant was prepared in advance in a biological safety cabinet (BSC). The cryoprotectant consisted of 50 percent HSA, 12 percent Hetastarch in Lactated Ringers solution, and 10 percent DMSO. This resulted in a final DMSO concentration

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of five percent in the final product. Once prepared, the solution was stored at 2-6 degrees C till needed.

Each collected product was transported from the collection area of the hospital to the processing laboratory using an insulated room temperature transport cooler. All products were cryopreserved within five hours of collection.

The weight of each HPC product was documented. The product was placed in a biological safety cabinet (BSC) and 1.5 ml of sample was removed for testing. At that point, a complete blood count (CBC), ABO type, differential, CD34 assay, culture, and viability were performed.

In order to cryopreserve the product, each fresh product was concentrated to have a maximum white blood count (WBC) of  $200 \times 10^6$  WBC/ml. The amount of excess plasma to remove was calculated using the product WBC count and the product weight to determine a concentrated product volume. The concentrated volume was subtracted from the product's original volume. This amount equaled the amount of plasma to be removed from the product.

To prepare the product for concentrating, it was transferred from the original container into a 600 ml sterile transfer pack. This 600 ml transfer pack was sterile docked to a 300 ml transfer pack. The product was placed inside a centrifuge bag, and centrifuged at 1800 rpms for 15 minutes. Once the centrifugation process was complete, the product was carefully removed from the centrifuge and placed on a plasma expresser. At this point, the appropriate calculated volume of plasma was removed. A WBC was performed on the final product and a WBC percent recovery calculated. Once a WBC recovery of greater than 80 percent was determined to have been achieved, the plasma was discarded.

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To prepare the concentrated product for cryopreservation, it was placed in a 2-6 degrees C refrigerator for 15-30 minutes. The cooled product and cryoprotectant were placed in a BSC. The appropriate amount of product was removed from the transfer pack using a syringe. A volume of cryoprotectant equal to the volume of the product was removed using a syringe. The product was next added to a cryocyte bag (Baxter), followed by the cryoprotectant. The product was constantly mixed during the addition of the cryoprotectant. Each individual unit was placed inside a metal canister and placed in a -95 degrees C mechanical freezer within 15 minutes of addition of cryoprotectant. When placed in the -95 degrees C freezer, each unit was placed between two pieces of one inch thick Styrofoam. The product was then left undisturbed for a minimum of 24 hours. After the initial 24 hours, each unit was placed into permanent storage in a -180°C vapor phase liquid nitrogen freezer.

## **QUALITY CONTROL**

Each product had quality control tests performed both pre-cryopreservation and post-cryopreservation in order to ensure product efficacy. Pre-cryopreservation testing included a CBC, CD34 assay performed using a flow cytometer, and 7-AAD viability on the flow cytometer. Cultures were also performed on pre-processing, post-processing, and cryoprotectant to ensure product sterility. Post-cryopreservation quality control testing includes trypan blue exclusion testing to determine post cryopreservation viability.

All transplants were autologous adult transplants. Different conditioning regimens were used and were determined on based on a patient by patient basis. The types of conditioning regimens can be seen in Table 2.

The day of transplant, each patient received appropriate pre-medication. The cryopreserved products were transported to the transplant unit on dry ice inside an insulated cooler. All cells were infused within five hours of leaving the processing facility. For infusion, each unit was thawed in a sterile zip lock bag in a 37 degrees C water bath. Once thawed, the product was transfused by gravity to the patient through a central venous line. Although most patients (n=11) received all transplant units in one day, a few patients received their units over a longer period (n=4 for two consecutive days, n=1 Boost transplant).

## **RESULTS**

Fifteen patients underwent autologous HPC, Apheresis transplants between March of 2008 and December 2008, for a total of 52 collections. Before beginning to cryopresere transplant products with cryoprotectant containing a final concentration of five percent DMSO, a comparison was performed to compare a previous formula consisting of a final concentration of 10 percent DMSO formula to the five percent DMSO formula. Initially, post-thaw viabilities were performed on samples cryopreserved and stored at -95 degrees C and compared to post-thaw viabilities on samples cryopreserved at -95 degrees C and then stored at -180 degrees C. The samples stored at -180 degrees C were moved after the sample had reached the -95 degrees C for 24 hours. This was done to show the five percent DMSO concentration would protect the cells in extreme cold. The results can be seen in Table 3.

Samples were cryopreserved using both the cryoprotectant consisting of a five percent DMSO concentration and of a 10 percent DMSO concentration. The comparison can be seen in Figure 1. The average viability of the 10 percent DMSO was 88.87 percent and the average viability of the five percent DMSO was 93.86 percent.

Cryopreservation of HPC products using the five percent DMSO concentration began. A total of 52 collections were performed and cryopreserved. The average peripheral WBC count  $\times 10^6$ , product volume, product  $\text{WBC} \times 10^6$ , hematocrit, red blood cell volume, product platelet count, CD34%, Total  $\text{CD34} \times 10^6$ , and the  $\text{CD34} \times 10^6/\text{kg}$  dose were calculated. The results are in Table 4.

The engraftment of the patients transfused with products cryopreserved with a 10 percent DMSO concentration were compared to patients who were transfused with products cryopreserved with a five percent DMSO concentration. The results of the platelet engraftment time, absolute neutrophil count (ANC) engraftment time, and the  $\text{CD34} \times 10^6/\text{kg}$  dose for patients transfused with the products containing a five percent DMSO concentration are shown in Table 5. The criteria for ANC engraftment is an ANC of  $>500/\text{ul}$  for three consecutive days. The criteria for platelet engraftment is a platelet count on the first of three consecutive days when the platelet count is  $>20,000$  without platelet transfusion support. The average dose given was  $4.557 \times 10^6$   $\text{CD34}/\text{kg}$ , an average ANC engraftment of 11.67 days, and an average platelet engraftment of 14.07 days.

## **DISCUSSION**

This study shows and investigation of a new cryopreservation procedure involving non-controlled rate freezing with final storage at either  $-95$  degrees C or at  $-180$  degrees C. A cryoprotectant consisting of a final DMSO concentration of five percent and including 25 percent HSA, and six percent Hetastarch in Lactated Ringers solution was used. A total of 52 collections were transfused to 15 different patients. The average dose received was  $4.557 \times 10^6$   $\text{CD34}/\text{kg}$  (range  $2.211$ - $5.277 \times 10^6$   $\text{CD34}/\text{kg}$ ). The average days until engraftment were 11.67 days

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(range 10-14) for ANC engraftment and 14.07 days (range 10-30) for platelets. There was only one case of mortality which was not caused by transplant related complications.

The engraftment rates for the year prior to the study, using the 10 percent DMSO concentration were 10.4 days for the ANC to reach >500/ul and the 14.6 days for the platelets to reach >20,000. When the five percent DMSO formula engraftment rates are compared to the 10 percent DMSO formula there was no significant difference. Engraftment rates were also compared to those of other clinical studies. The range of averages from these studies was 10-11.5 days for ANC engraftment and 11-13.90 days for platelets<sup>1,3,7,10,11</sup>. Although each clinical varied in cryopreservation method, there was no significant difference in engraftment rates when compared.

In conclusion, there was no significant difference in viability or engraftment when peripheral blood HPC products were cryopreserved at -95 degrees C using the cryoprotectant consisting of a final DMSO concentration of five percent and including 25 percent HSA and six percent Hetastarch in Lactated Ringers solution. This formula allows a decreased processing cost and an alternative to facilities which do not have access to a controlled rate freezer. Products may also be stored in vapor or liquid phase liquid nitrogen without affecting the cells. Most importantly, this method of cryopreservation results in unaffected hematological engraftment, and provides a product with a lower DMSO concentration reducing the DMSO toxicity to the patient without adversely affecting the cells.

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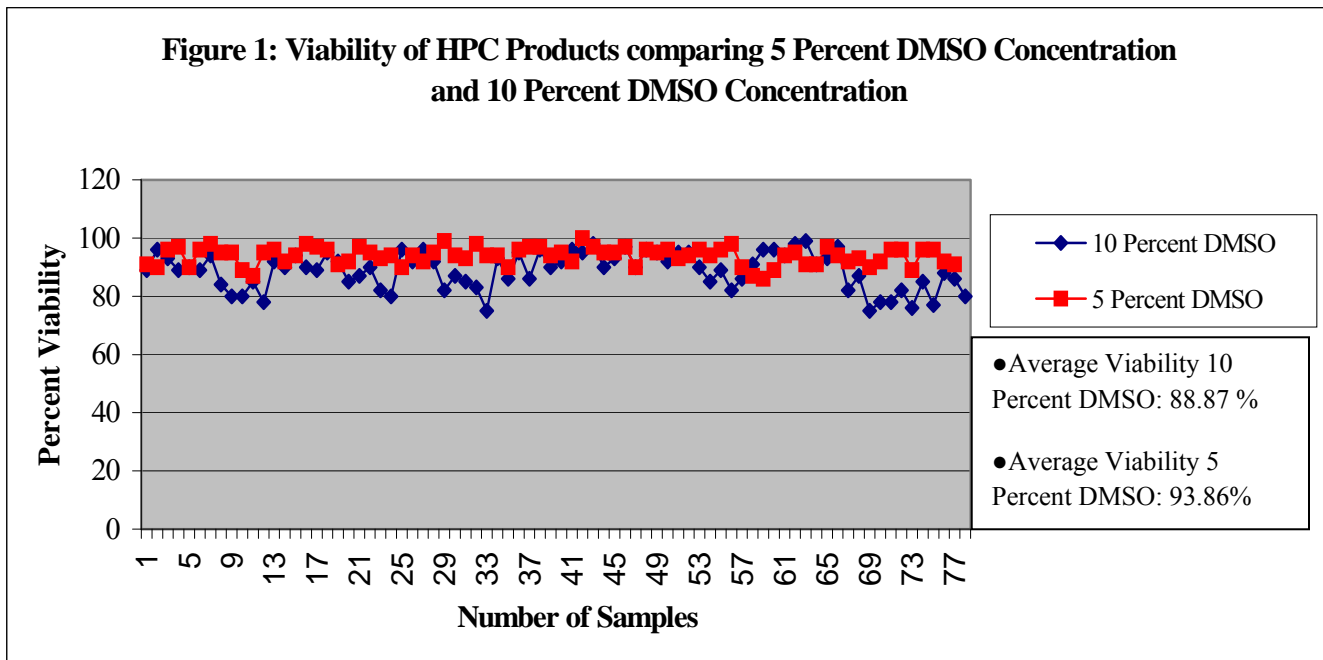
**Table 1. Patient Characteristics:**

Number of patients:	15
Diagnosis	
Multiple Myeloma	6
Large B-Cell Lymphoma	2
Non-Hodgkins Lymphoma	3
Mantel Cell Lymphoma	1
Amyloidosis	1
Plasmacytoma	1
T- Cell Lymphoma	1

**Table 2. Patient Conditioning Regimens**

Condition Regimen	Number of Patients
BEAM	6
Melphalan	8
CBV	1

Sample	Storage	Viable Cells	Dead Cells	% Viability
1	-95	165	19	89.67
2	-95	179	22	89.05
3	-180	179	22	89.05
4	-180	177	12	93.22



**Table 4. Product Information (n=52)**

Test	Range	Average
Peripheral WBC count x10 <sup>6</sup>	1.2-81.3	23.1
Product Volume (ml)	183.4-399.4	249.7
Product WBC x 10 <sup>6</sup> /ml	21.26-287.26	127.36
Product HCT	2.0-8.5	4.1
Product RBC Volume (ml)	5.2-24.4	10.1
Collected Product Platelet	181-3240	685.1
Product CD34 %	0.013-2.331	0.496
Product Total CD34 Cellsx10 <sup>6</sup>	5.743-998.297	128.616
Patient Dose in CD34 x10 <sup>6</sup> /kg	0.077-15.382	1.595

**Table 5. Engraftment and Average Dose**

Patient	Dose(CD34x10 <sup>6</sup> /kg)	ANC Engraftment(Days)	Platelet Engraftment(Days)
1 expired	2.413	13	No engraftment, patient
2	5.121	12	12
3	4.847	11	12
4	4.940	12	16
5	4.649	12	10
6	2.291	12	11
7	3.159	14	14
8	3.506	11	30
9	2.991	12	12
10	3.487	11	13
11	5.174	11	12
12	2.586	11	12
13	15.382	10	11
14	5.277	11	16
15	2.535	12	16
Average	4.557	11.67	14.07