

Cryopreservation of Hematopoietic Progenitor Cells (HPC) Apheresis using 5% Dimethyl Sulfoxide without Hydroxyethyl Starch

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Abstract:

Cryopreservation of human HPC derived from apheresis (HPC-A) and bone marrow (HPC-M) is widely performed using the established cryoprotectant containing 5% dimethylsulfoxide (DMSO), 6% hydroxyethyl starch (HES), 0.2% dextrose and 3.75% human serum albumin (HSA) in Normosol-R™ [DMSO/(+)HES] (Stiff et al, 1987, Blood 70:974-978). However, the use of HES in the clinical setting requires investigational new drug (IND) approval and reporting in the US and involves the in-house manufacture and release testing of the DMSO/(+)HES, which can be time consuming, variable and costly. Access to GMP-grade HES can also be problematic.

In an attempt to determine the necessity for the HES component of the cryoprotectant for cryopreservation of HPC-A, which was originally adapted by Stiff et al for the cryopreservation of HPC-M mononuclear cells in a -86°C mechanical freezer (MF) (dump freezing) without a controlled rate freezer (CRF), we evaluated various freezing and cryostorage procedures for HPC-A using cryoprotectant formulated with 5% DMSO and 2.25% HSA in saline without HES [DMSO/(-)HES]. HPC-A products were obtained by leukapheresis from non-mobilized, normal donors and were cryopreserved with 1:1 volume of DMSO/(-)HES or DMSO/(+)HES as controls to achieve a final DMSO concentration of 5% in 70-ml cell suspension loaded in 250-ml Cryocyte Freezing Containers (Baxter, R4R9953). HPC-A products were either dump frozen in the -86°C MF or through the use of a CRF and were cryostored for two to seven days in either the -86°C MF or the vapor phase (< -135°C) of liquid nitrogen (VLN) before thawing for cell quality assessments.

Cell viability as determined by trypan blue (TB) exclusion assay for samples cryopreserved using DMSO/(-)HES were ≥ 90% for all combinations of cryoprotectant, cryopreservation and storage procedures used. However, viability determination using 7-amino-actinomycin D (7-AAD) by flow cytometry showed a slight, but not significant reduction (P=0.23 and 0.09 by comparing the sample groups frozen in MF and CRF, respectively, using paired t-Test analyses) in viability of the CD45+ cell population for the samples cryostored in -86°C MF (86±8% and 87±2% frozen in MF and CRF, respectively) as compared to those in VLN (94±1% and 93±2% frozen in MF and CRF, respectively). The recovery of CD34+ cells (determined by flow cytometry) and hematopoietic colony forming unit (CFU) revealed a similar trend, as the recovery of both cell populations from the pre-frozen samples were lower in samples stored in a MF (CD34+ cell recovery of 87±25% and 79±11%; and CFU recovery of 72±8% and 63±7% for samples initially frozen in MF and CRF, respectively) than in LNF (CD34+ cell recovery of 102±44% and 96±35%; and CFU recovery of 104±44% and 116±97% for samples initially frozen in MF and CRF, respectively) but again such differences were not significant (P=0.51 and 0.35 for CD34+ cell recovery and P=0.36 and 0.44 for CFU recovery by comparing the sample group frozen in MF and CRF respectively).

In comparison, TB viability, 7-AAD viability, CD34 recovery and CFU recovery of HPC-A cryopreserved using DMSO/(+)HES frozen and cryostored in a -86°C MF were 98±1% 96±1%, 94±18% and 103±9%, respectively.

In conclusion, 5% DMSO and 2.25% HSA in saline appears to be a suitable alternative cryoprotectant for cryopreservation of HPC-A for clinical use. HPC can be frozen either by MF or CRF with this cryoprotectant, but cell quality is likely better preserved in VLN for longer term storage. Further studies to assess patient engraftment efficiency of the infused HPC-A cryopreserved with this cryoprotectant are in progress.

Introduction:

Human hematopoietic progenitor cells (HPC) derived from apheresis (HPC-A) and bone marrow (HPC-M) are routinely cryopreserved until they are used for transplantations. Cryoprotectant formulated with 5% dimethylsulfoxide (DMSO), 6% hydroxyethyl starch (HES), 0.2% dextrose and 3.75% human serum albumin (HSA) in Normosol-R™ [DMSO/(+)HES], originally adapted by Stiff et al (1987) for cryopreservation of mononuclear cells from bone marrow in a -86°C mechanical freezer (MF) (dump freezing) without a controlled rate freezer (CRF), is commonly used for cryopreserving these cells for clinical applications. However, use of HES for cryopreserving clinical HPC products requires IND approval and reporting in the US. As no formulated GMP-grade DMSO/(+)HES is currently commercially available, the preparation of such cryoprotectant requires in-house manufacture and release testing, which can be time consuming and costly.

In this study, we evaluated a cryoprotectant formulated with 5% DMSO and 2.25% HSA in saline without the inclusion of HES [DMSO/(-)HES] as an alternative cryoprotectant for cryopreservation of HPC. The cell quality of HPC-A following cryopreservation with this cryoprotectant under various freezing and cryostorage conditions were compared.

Methods:

Preparation of cryoprotectant formulations:

- A. DMSO/(+)HES: 250-ml of sterile 2x concentrated solutions in glass bottle containing 10% DMSO (Edwards Life Sciences, Cryoserv), 12% HES (B. Braun), 0.4% dextrose (Fisher) and 7% HSA (Baxter) in Normosol-R™ pH 7.4 (Abbott Laboratories) were prepared under cGMP conditions according to the SOP established at the Progenitor Cell Therapy, LLC.
- B. DMSO/(-)HES: 2x concentrated solution was prepared aseptically by adding 56-ml of DMSO in a 500-ml bottle of 5% HSA in saline (Baxter 060-049). The prepared solution contains 10% DMSO and 4.5% HSA.

Cryoprotectants were kept refrigerated (2-8°C) until used.

HPC-A collection:

Healthy donor leukaphereses were collected (5-8 liters of blood processed) using a COBE Spectra® Apheresis System at the Apheresis Center of the Hackensack University Medical Center. 3 units of HPC-A were used to complete this study.

Cryopreservation of HPC-A product:

The collected HPC-A units were processed within 2-h from collections. All steps of processing were performed in various sizes of transfer pack containers (Baxter/Fenwal). HPC-A was pooled with the autologous plasma and was diluted with Normosol-R pH7.4 to yield sufficient volume for validating all test conditions. For cryopreservation, diluted HPC-A was mixed with an equal volume of chilled 2x concentrated cryopreservative media [either DMSO/(+)HES or DMSO/(-)HES]. After mixing, the HPC-A samples would have final concentration of 5% DMSO, 6% HES, 0.2% dextrose and 3.75% HSA if DMSO/(+)HES cryoprotectant was added or 5% DMSO and 2.25% for DMSO/(-)HES. 70-ml of HPC-A/cryoprotectant mixture was loaded into a 250-ml Cryocyte freezing containers (Baxter, R4R9953). Additionally, cell suspensions (1.8-ml) were frozen in retain cryogenic vials (Coming). Samples were frozen within 30 minutes from the addition of cryoprotectant either by dump freezing in a -86°C mechanical freezer (MF) (Revco ULT 2586-A36) or program freezing (Table 1) in a controlled-rate freezer (CRF) (Custom Biogenics Systems 2100). Cryostorage of frozen HPC samples was accomplished in either the -86°C MF or the vapor phase (< -135°C) of liquid nitrogen (VLN) (MVE Cryogenics XLC-1814) for a duration of 2-7 days before thawing at 37°C in the water bath for cell quality assessments.

ion and conclusion:

- A. While the viability of HPC-A cryopreserved in 5% DMSO and 2.25% HSA under various freezing and storage condition was ≥ 90% as determined by trypan blue assay, the sample group that cryostored in a -86°C mechanical freezer versus that in vapor phase of liquid nitrogen showed a slight reduction in viability as determined by 7-AAD flow cytometry (Table 2).
- B. The recovery of both CD34+ cells and CFU were also slightly reduced in samples cryostored in the mechanical freezer than in vapor phase of liquid nitrogen (Table 2).
- C. However, all these observed differences were not statistically significant (P>0.05) as determined by paired t-Test.
- D. The cell quality of HPC-A sample group cryopreserved with 5% DMSO and 2.25% in the vapor phase of liquid nitrogen was comparable to that with the HES formulated cryoprotectant (Table 2).
- E. Traditionally, in addition of cryopreserving the HPC products in freezing bags, cryopreservation of small volume of the products in retain cryogenic vials would be performed which are intended to be used to represent the contents of the freezing bags. Table 3 shows the viability of the cryopreserved samples in cryogenic vials under various freezing and cryostorage conditions are comparable with those cryopreserved in the freezing bags.
- F. In conclusion, 5% DMSO and 2.25% HSA in saline appears to be a suitable alternative cryoprotectant for cryopreservation of clinical HPC products. HPC can be frozen by dump freezing in a -86°C mechanical freezer or by controlled-rate freezing with this cryoprotectant, however this study showed that HPC quality is likely better preserved in the vapor phase of liquid nitrogen for longer term storage.

Table 1. Freezing temperature program for freezing HPC-A samples in controlled-rate freezer.

Sequence of temperature control	Target temperature (°C)	Rate (°C per min)	Temperature monitoring
1	4	10	Chamber
2	-4	5	Sample
3	-86	60	Chamber
4	-35	15	Chamber
5	-35	5	Sample
6	-86	10	Sample

Sample testing:

Samples were taken from the HPC-A before the addition of cryoprotectant and after cryopreservation for testing. The following assays were performed on each sample prepared:

- A. Nucleated cell viability was determined by trypan blue (TB) exclusion assay.
- B. Viability of CD45+ cell population was determined by 7-amino-actinomycin D (7-AAD) exclusion, a flow cytometry assay combined with CD34+ cell enumeration (See below).
- C. CD34+ cell enumeration by flow cytometry using the Stem-Kit™ (Beckman Coulter IM3630) was performed essentially as described by Sutherland et al (1996).
- D. Colony forming unit (CFU) growth in MethoCult™ GF H4434 Medium (Stem Cell Technologies) was performed following the procedure adapted by Miller and Lai (2005).

Results:

Table 2. Cell quality assessments of HPC-A following cryopreservation with cryoprotectant formulated with or without hydroxyethyl starch under specific freezing and cryostorage conditions.

Cryoprotectants	Freezing and cryostorage conditions	Viability (% ± SD) ⁸		Recovery ⁹ (% ± SD) ⁹	
		Nucleated cells (Trypan blue assay)	CD45 ⁺ Cells (7-AAD assay)	CD34 ⁺ cells	CFU
Pre-cryopreservation ¹	-	100 ± 0	97 ± 4	-	-
DMSO/(+)HES ²	MF ⁴ -MF ⁶	98 ± 1	96 ± 1	94 ± 18	103 ± 9
DMSO/(-)HES ³	MF-MF	90 ± 6	86 ± 8	87 ± 25	72 ± 8
	MF-VLN ⁷	95 ± 1	94 ± 1	102 ± 44	104 ± 44
	CRF ⁵ -MF	90 ± 1	87 ± 2	79 ± 11	63 ± 7
	CRF-VLN	96 ± 2	93 ± 2	96 ± 35	116 ± 97

¹ HPC-A samples taken before the addition of cryoprotectant

² Cryoprotectant formulated with 5% DMSO, 3.75% HSA, 0.2% Dextrose and 6% Hydroxyethyl Starch in Normosol-R solution, pH7.4

³ Cryoprotectant formulated with 5% DMSO and 2.25% HSA in saline

⁴ Freezing condition: Dump freezing in -86°C mechanical freezer

⁵ Freezing condition: Program freezing in controlled rate freezer

⁶ Cryostorage in a -86°C mechanical freezer

⁷ Cryostorage in vapor phase of liquid nitrogen

⁸ Standard deviation of the results from 3 independent runs

⁹ % difference of CD34⁺ cells or CFU between cryopreserved HPC-A and the HPC-A before the addition of cryoprotectant

Table 3. Viability of HPC-A in cryogenic vials following cryopreservation with cryoprotectant formulated with or without hydroxyethyl starch.

Cryoprotectants	Freezing & cryostorage conditions	Viability of HPC-A cryopreserved in cryogenic vial (% ± SD) (Trypan blue assay)
Pre-cryopreservation	-	100 ± 0
DMSO/(+)HES ²	MF-MF	98 ± 1
DMSO/(-)HES	MF-MF	90 ± 4
	MF-VLN	93 ± 4
	CRF-MF	94 ± 5
	CRF-VLN	94 ± 5

Discussion and conclusion:

- A. While the viability of HPC-A cryopreserved in 5% DMSO and 2.25% HSA under various freezing and storage condition was ≥ 90% as determined by trypan blue assay, the sample group that cryostored in a -86°C mechanical freezer versus that in vapor phase of liquid nitrogen showed a slight reduction in viability as determined by 7-AAD flow cytometry (Table 2).
- B. The recovery of both CD34⁺ cells and CFU were also slightly reduced in samples cryostored in the mechanical freezer than in vapor phase of liquid nitrogen (Table 2).
- C. However, all these observed differences were not statistically significant (P>0.05) as determined by paired t-Test.
- D. The cell quality of HPC-A sample group cryopreserved with 5% DMSO and 2.25% in the vapor phase of liquid nitrogen was comparable to that with the HES formulated cryoprotectant (Table 2).
- E. Traditionally, in addition to cryopreserving the HPC product in freezing bags, cryopreservation of a small sample volume of product in retain cryogenic vials would be performed. This sample is intended to be used to represent the contents of the freezing bags. Table 3 shows the viability of the cryopreserved samples, in cryogenic vials under various freezing and cryostorage conditions, is comparable with those cryopreserved in the freezing bags.
- F. In conclusion, 5% DMSO and 2.25% HSA in saline appears to be a suitable alternative cryoprotectant for cryopreservation of clinical HPC products. HPC can be frozen by dump freezing in a -86°C mechanical freezer or by controlled-rate freezing with this cryoprotectant, however this study showed that HPC quality is likely better preserved in the vapor phase of liquid nitrogen for longer term storage.
- G. Further studies to evaluate the cell quality of HPC-A for long term cryopreservation and the patient engraftment efficiency of the transplanted HPC-A cryopreserved with this cryoprotectant are in progress.

References:

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