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Outcome of 51 autologous peripheral blood stem cell transplants after uncontrolled-rate freezing ("dump freezing") using −80°C mechanical freezer

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Abstract:

BACKGROUND AND OBJECTIVE: Controlled-rate freezing is a complicated, expensive, and time-consuming procedure. Therefore, there is a growing interest in uncontrolled-rate freezing (UCF) with −80°C mechanical freezers for cryopreservation of hematopoietic stem cells. This is a retrospective analysis of efficiency of UCF and outcome of autologous peripheral hematopoietic stem cell (PBSC) transplants at our center from December 2011 to June 2016.

MATERIALS AND METHODS: Cryoprotectant solutions used included 5% dimethyl sulfoxide and 5% albumin with 2% hydroxyethyl starch and stored at −80°C mechanical freezer till transplant. Evaluation of cryopreservation was studied by analyzing the variation in cellularity, viability, and CD34+ stem cell dose recovery as well as clinical follow-up with engraftment.

RESULTS: A total of 51 patients (23 females and 28 males) underwent autologous PBSC transplantations with a median age of 31 years (range: 3–60 years) for both hematological and nonhematological indications. Mean recovery post by UCF at −80°C mechanical was 92.9% ± 15.5% for nucleated cells, 86.6% ± 15.5% for viability, and 80% ± 21.5% in CD34+ dose. The median day to neutrophil engraftment was 10 (range 5–14 days) and platelets engraftment was 15 (range 8–45 days). The cryopreserved products were stored at −80°C for median 7 days (range 2-41 day) before transplant.

DISCUSSION/CONCLUSION: Our analysis shows that PBSC can be successfully cryopreserved with mechanical uncontrolled rate freezing. This is a cheap and simple method to freeze the stem cells for a short period in resource-constrained setting.

Keywords:

Cryopreservation, dump-freezing, hematopoietic stem cells, uncontrolled-rate freezing

Introduction

Controlled-rate freezing (CRF) is the most widely accepted method for cryopreservation of hematopoietic progenitor stem cells for both hematological and solid organ transplants.[1] CRF is complicated, expensive, and time-consuming procedure requiring elaborate infrastructure with high establishment costs; hence, there is a growing interest in uncontrolled-rate freezing (UCF) at −80°C using mechanical freezers, also known as "dump freezing." Several studies[2-14] have demonstrated that CRF is not an absolute requirement for a successful cryopreservation of progenitor cells, and dump freezing offers more better

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economical option for cryopreservation (up to 6 months) in resource-constrained settings. In this study, we retrospectively analyzed the efficiency and outcome of UCF used for cryopreservation of peripheral blood hematopoietic stem cells for hematological and solid organ malignancies.

Materials and Methods

This study is a retrospective analysis of outcome of autologous transplants of peripheral hematopoietic stem cells (PBSCs) following UCF cryopreservation at our center from December 2011 to June 2016. Efficiency of UCF mode of cryopreservation was also analyzed as a part of the study.

Patients characteristics

Patients, who underwent autologous PBSC transplantations, by UCF cryopreservation for various hematological and nonhematological indications, were analyzed. All the patients were provided with the informed consent regarding the autologous PBSC harvest and cryopreservation.

Graft harvest and characteristics

Mobilization and collection of peripheral hematopoietic stem cells

For mobilization of PBSC, we used human granulocyte-colony-stimulating factors (GCSF; 10 μg/kg/day single doses) administered subcutaneously (SC) starting 5 days before leukapheresis. A circulating CD34+ cells level >10 × 10^6/L or total white blood cells count >20 × 10^9/L was used as the main criterion for the beginning of leukapheresis. In case of poor mobilization, plerixafor (0.24 mg/kg SC) was added to the mobilization regimen on day 5 or 6 with GCSF. In cases of inadequate collection of CD34+ cell dose on the first day of collection, another session of leukapheresis was done on the next day until the target dose was achieved.

All leukapheresis procedures for mononuclear cell collection were performed with two cell separators (COBE spectra, Gambro BCT, Bourg-la-Reine, France) and Amicus separator system (Fresenius Kabi, USA). In case the weight of the patient was <20 kg, a compatible irradiated leukoreduced packed red blood cell was used for priming the cell separators before connecting to the patients.

Freezing and thawing methods

Each leukapheresis mononuclear cell collection product was cryopreserved with a simplified cryopreservation method using −80°C mechanical freezers with laminar hood [Figure 1]. Cryoprotective solutions (CSs) included dimethyl sulfoxide (DMSO) (99.9%, CryoSure-DMSO; WAK-Chemie Medical GmbH, Germany), 20% Human Albumin (Baxter), and 6% hydroxyethyl starch (HES) (Voluven, Fresenius Kabi, Sevres, France).

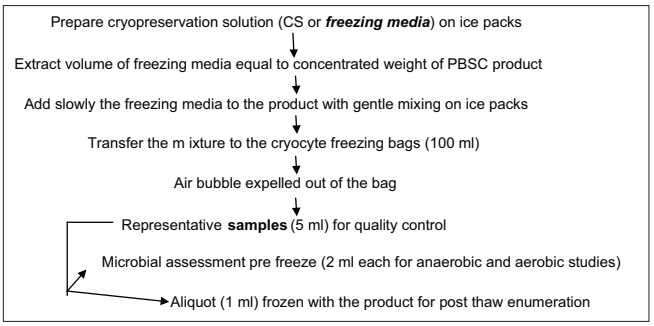

Figure 1: Uncontrolled-rate freezing (UCF) protocol for cryopreservation for PBSC

CSs were mixed with the PBSC product at 4°C on ice packs under sterile conditions using a laminar hood. Volume of cryoprotectant solution used was equal to the volume of PBSC product collected (e.g., 150 ml of CS was used for preserving 150 ml of product). Final concentration of cryoprotectants were 5% for DMSO, 5% albumin, and 2% HES in the final mixture. After mixing at 4°C, PBSC with the CS solution was quickly transferred into Cryostore Freezing Bags (Origen BIOMEDICAL; Schwaig or Macopharma's EVA bags) and transferred into −80°C mechanical freezer (Thermo, Saint Herblain, France). The maximum volume stored in each bag was 100 mL.

At the same time, an aliquot of 5 mL was sent for microbiological assessment and an aliquot of 1 mL was stored in polypropylene vials with each bag at −80°C. To achieve a uniform heat exchange and bag thickness, all bags and vials were sandwiched between standard freezing aluminum plates cassette for freezing and placed in the −80°C mechanical freezer. Mechanical freezer was used for storage until transplantation. Continuous temperature monitoring was done for the mechanical freezer both by thermographs as well as manually.

Volume of the apheresis product to be cryopreserved was adjusted (reduced) by plasma reduction to reduce the exposure to DMSO. Volume reduction was done by plasma removal after centrifuging with Cryofuge 6000i (Thermo) at 2000 rpm for 10 min at 4°C–6°C. Pre-UCF counts were done postproduct manipulation and before addition of CS.

For the transplant, the frozen bags were rapidly immersed in 37°C sterile water bath for thawing and infused to the patient through central venous catheters. The samples stored in vials were sent for stem cell enumeration and blood counts to evaluate the postcryopreservation cell dose. Patients did not receive growth factors posttransplantation.

Evaluation of cryopreservation

Efficiency of cryopreservation was studied by analyzing the variation in cellularity, viability, and stem cell recovery by mechanical freezing. Precryopreservation (apheresis product) and postthawing samples (analyzed as it is postthaw without any washing or modification) were compared and statistically analyzed using paired *t*-test [Table 1]. Evaluation was done by calculating the recovery (mean ± standard deviation [SD]) of total nucleated cells (TNCs), absolute mononuclear cell counts, absolute CD45+ cell counts, and absolute CD45+ and CD34+ cell counts postthawing. Viability of MNCs after thawing was done by estimation of 7-aminoactinomycin-D (7-AAD) in flow cytometric analysis.

Blood cell counts such as TNC were done using LH750 Beckman Coulter (Florida, Miami, USA). Viability, absolute mononuclear cell count, and CD45+ and CD34+ cell count were done using BD FACS Canto-II Flow-Cytometer. Enumeration of CD34+ cells was done by flow cytometry as described by International Society of Hematology and Graft Engineering guidelines.[15]

Engraftment and clinical follow-up

Successful engraftment was defined as 1st day of three consecutive days on which neutrophil count exceeded 0.5×10^9/L and platelets exceeding 20×10^9/L without platelet transfusion during a 7-day period. Delayed engraftment was considered when required engraftment time was more than 3 months. All the patients received supportive care during the pre- and post-transplant period. Overall survival was calculated from the date of transplant to last follow-up (June 31, 2016). In case of mortality, the cause of death was analyzed.

Results

A total of 51 patients (23 females and 28 males) underwent autologous PBSC transplantations for various hematological and nonhematological indications. The median age of the patients transplanted was 31 years (range: 3–60 years). Demography of patients, indication for transplant, disease status at the time of transplant, and conditioning regimen used are discussed in Table 2.

Graft harvest

Adequate PBSCs mobilization was achieved in 43 of 51 patients by GCSF as a sole mobilizing agent alone. In eight GCSF alone poor mobilizers, plerixafor was added in the mobilizing regimen. Days required for adequate mobilization and collection from initiation of mobilization were 5 days (median; range 4–7 days). Twenty-three patients mobilized and completed the adequate dose of CD34+ cells on 1st day of harvest, whereas 27 patients required a second day and 1 patient required 3 days. Out of 28 requiring additional day to complete the harvest, 8 required plerixafor before the second harvest. In total 80 PBSC harvests, COBE Spectra was used in 23 patients (35 harvest procedures), and Amicus in other 28 patients (45 harvest procedures) was done. Mean 3.9 times (range: 3-5.5 times) of blood volume was processed for each harvest.

Volume of leukapheresis product harvested was 372.3 ± 158.1 ml (mean ± SD). Plasma volume reduction of 251.4 ± 153.1 ml was done to achieve final hematopoietic stem cell volume to 119.7 ± 17.7 ml. CSs added were 120.5 ± 18.1 ml to cryopreserve the product. The final volume of the mixture to be cryopreserved achieved was 240.2 ± 35.4 ml.

Evaluation of cryopreservation by uncontrolled-rate freezing and transplant

The cryopreserved product was stored at −80°C for median 7 days (range 2–41 day) before transplant. The products were thawed at 37°C at the bedside and transplanted as soon as possible upon thawing. One of the patients reported to have a severe anaphylactic reaction to DMSO but recovered. Other adverse reaction observed during transplantation of these thawed products included headaches, chills, dyspnea, cough, and a few patients had hypotension.

Mean recovery and effect of cryopreservation by UCF at −80°C is discussed in Table 1. There was a statistically significant reduction in the viability (7-AAD), absolute CD45+ cells, and absolute CD34+ cells by the process of cryopreservation.

Table 1: Evaluation of efficiency of uncontrolled-rate freezing method cryopreservation

Parameter	Pre-UCF	Post-UCF	Recovery (%)	*P*
Mean absolute nucleated cell (ANC; $\times 10^9$)	71.1±47.5	61.8±27.8	92.9±15.5	0.21
Mean absolute mononuclear cell count ($\times 10^9$)	55±37.1	56.3±35.5	150.94±302	0.6
Mean absolute CD45+ cells ($\times 10^9$)	73.4±53.1	51.6±46.2	83.8±101.5	0.02
Mean absolute CD45+/CD34+ cells ($\times 10^6$)	280.9±204.1	229.3±178.6	129.69±38.8	<0.01
Mean viability (7-AAD; %)	96.0±2.5	80.4±15	86.6±15.5	<0.01
Mean CD34+ cell dose (10^6/kg/L)	4.6±2.5	3.6±2.1	82±21.5	<0.01

Mean±SD shown; Paired *t*-test used for calculating *P* value; "pre-UCF" is the leukapheresis product cell count and "post-UCF" is the post thaw analysis.
SD = Standard deviation, UCF = Uncontrolled-rate freezing, 7-AAD = 7-aminoactinomycin-D, ANC = Absolute mononuclear cell

Table 2: Demographic details of the patients analyzed

Number of Patients/Transplant Analyzed	Total (N=50)
Number of patients/transplants analyzed	51
Median age (range)	31 (3-60 years)
Gender (female/male)	23/28
Diagnosis	
Lymphoma	39
Leukemia (APML)	3
Myeloma	1
Neuroblastoma	4
Ewing's sarcoma	1
Scleroderma	2
Disease status at transplant (%)	
Active disease	2 (4)
CR, PR	47 (92)
PD	2 (4)
Conditioning regimens (%)	
Nonmyeloablative regimen	3 (6)
Myeloablative regimens	48 (94)

APML = Acute promyelocytic leukemia, CR = Complete remission, PR = Partial remission, PD = Progression

Engraftment and clinical follow-up

Out of 51 patients under our retrospective analysis, there were six transplant-related mortalities. Out of the six mortalities, two did not engraft (day 5 and day 6), one engrafted partially (only neutrophil engraftment; day 14), and three engrafted completely (day 20, day 24, and day 244) but all of them succumbed to infections in the posttransplant phase.

Forty-five patients who are alive with a median follow-up of 566 days (range 11–1624 days) achieved complete neutrophil and platelet engraftment. Median days to neutrophil engraftment were 10 (range 5–14 days) and platelet engraftment were 15 (range 8–45 days).

Discussion

Our retrospective analysis shows that UCF with mechanical freezers can be safely used for cryopreservation of PBSC harvest for autologous transplants. Many previous studies [Table 3] have also shown that autologous PBSC harvest can be stored at −80°C with optimal stem cell recovery. One of the most initial attempts was by Stiff *et al*.[2] who was able to store stem cells with DMSO, HES, and albumin with −80°C mechanical freezers with 9% loss of nucleated cells.

Cryopreservation procedure causes harm to the hematopoietic progenitor cells due to direct injury from low temperature as well as due to the formation of intracellular ice crystals. Technique of cryopreservation involves mainly three areas which determine the outcome of the cells stored, namely CS used, method for freezing, and temperature of storage after freezing. Cryoprotectant solutions majorly include DMSO, which has been used to freeze red cells[16] initially. DMSO protects the integrity and viability providing colligative cryoprotection. DMSO penetrates the cells, reduces the water incorporation into the cell, and protects the cells from excessive dehydration. Nearly 10% DMSO is an optimal established concentration for preservation of hematopoietic stem cells although lesser concentrations have also been successfully applied for the transplants[17] and often recommended to avoid the adverse reactions related to the infusion of DMSO.

One of the studies by Galmes *et al*.,[12] compared toxicity and outcome of using 5% and 10% DMSO without HES for cryopreservation by UCF method. The study showed 5% DMSO had slower hematological recovery compared to 10% DMSO, despite receiving a higher number of cells at the time of transplant. The study also showed a marked reduction (about 60%) in infusion-related toxicity with 5% as compared with 10% DMSO. Hence, a lower concentration of DMSO has been suggested for cryopreservation of stem cells up to 6 months without significantly affecting the long-term hematological recovery.

Method of freezing or the rate of cooling is another aspect of successful preservations. One of the studies,[18] which compared controlled and UCF protocols, showed that the PBSCs can be collected, stored at 1°C–6°C for 24 h, and cryopreserved using 5% DMSO with 6% HES in mechanical freezers at −80°C. The rate of cooling of −80°C mechanical freezers ranged between 0.36°C and 1°C/min in the study and showed comparable results in terms of viability of the stem cells with decrease in CFU-GM clonality assay with UCF.

Guidelines for postthaw evaluation by flow cytometry of these cryopreserved products are not standardized yet,[19] and it accounts for a careful assessment of cells considering the effect of freezing, thawing, processing,[20] and use of cryoprotectants (e.g., DMSO and HES, etc.).[21,22] Our protocol included the assessment of the representative aliquot vial stored with each bag at −80°C; these aliquots were thawed at 37°C and evaluated immediately on flow cytometer based on the similar guidelines as the prefreezing samples were evaluated.

Postthaw assessments reported by various studies have shown a marked variation. Earlier studies documented viability using trypan blue dye whereas recent published literature shows the use of flow cytometry as a method to analyze viability in the product. This accounts for a careful comparison of the published literature with the current studies. We used commonly used dye to analyze membrane integrity using flow cytometry which is presently considered as a gold standard for analysis of these products.

Table 3: Efficiency and outcome of uncontrolled-rate freezing, method used by various authors

Authors	Stem cells source	Cryoprotectant solution			Cryopreservation		Time[a] in storage	Recovery (%)			Engraftment (median days with range)			
		DMSO (%)	HES (%)	Plasma/ albumin (%)	Freezing temperature (°C)	Storage temperature (°C)		Viability	TNC	CD34+	Neutrophil		Platelets	
											>0.5×10⁹/L	>1×10⁹/L	>20×10⁹/L	>50×10⁹/L
Stiff *et al*.[2]	BM	10	12	8	−80	−80	NR (1 week to 4 months)	82.2±9.2	96.7±11.06	NR	NR	21	12	NR
Clark *et al*.[3]	BM	10	NU	AP	−80	−80	5 weeks (2-13)	98-100	NR	NR	18 (17-20)	NR	23 (16-31)	NR
Makino *et al*.[4]	PBSC	5	6	AP	−80	−80	12 weeks (2-13)	>80	86.6±12.3	NR	14 (9-23)	NR	NR	18 (11-240)
Galmés *et al*.[5]	PBSC and BM	10	NU	AP	−80	−80	>13 weeks	90	86	NR	11 (7-18)	NR	NR	13 (4-21)
Galmés *et al*.[6]	PBSC	5	NU	AP	−80	−80	5 weeks (2-11)	84.7±100.6	86±7.9	NR	11 (10-15)	NR	NR	12 (9-22)
Galmés *et al*.[7]	PBSC	5	NU	AP	−80	−80	22 weeks (18-29)	78	91	NR	12 (10-27)	NR	15 (10-39)	NR
Choi *et al*.[8]	PBSC	5	NU	2.5	−80	LN	76 days (18-145)	NR	NR	95.3	8 (5-13)	31 (16-140)	27 (16-78)	NR
Halle *et al*.[9]	PBSC	3.5	2.5	1	−80	−80	7 weeks (1-78)	68	60.8	79.6	11 (0-37)	NR	11 (0-129)	NR
Montanari *et al*.[10]	PBSC	10	NU	2.5	−80	LN	NR	NR	NR	NR	11 (7-22)	NR	13 (8-39)	18 (10-95)
Kudo *et al*.[11]	PBSC	5	6	4	−80	−80	59 days (14-365)	90±6.2	NR	96.6±32.7	10.5	NR	15 (6-42)	NR
Galmes *et al*.[12]	PBSC	5	NU	AP	−80	−80	31 days (8-208)	NR	NR	NR	11 (6-34)	NR	17 (8-704)	NR
Iannalfi *et al*.[13]	PBSC	10	NU	AP	−80	−80	12 weeks (4-31)	NR	NR	NR	11 (8-14)	15 (10-23)	14 (9-45)	19 (12-52)
Calvet *et al*.[14]	PBSC	3.5	2.5	1	−80	−80	1.7 months (1-5.9)	NR	72	98.6	13 (1-43)	NR	12 (1-125)	14 (3-189)
Our study 2016	PBSC	5	2	5	−80	−80	7 days (2-41 days)	86.6	92.9	129.69	10 (10-14)	NR	15 (8-45)	NR

[a]Mean or median of days/week months reported with a range in the bracket; recovery is reported as the mean percentage. BM = Bone marrow, PBSC = Peripheral blood stem cells, AP = Autologous plasma, NR = Not reported, NU = Not used, LN = Liquid nitrogen, NC = Total nucleated cell counts, DMSO = Dimethyl sulfoxide, HES = Hydroxyethylstarch

Freezing and thawing induce significant membrane alterations, and often integrity measures of thawed cells poorly correlate with the postthaw functions.[23] Studies[24,25] have shown >100% recovery of cells postthaw, which can be due to the freezing process *per se*. Cryopreservationinduces alteration in membrane structure and can cause nonspecific binding of the antibodies (used for staining) as well as alters the optical properties of these membranes, hence due to change in shape and affinity with the dye postthaw samples often report higher recoveries. Due to different membrane contents (mainly lipids and proteins), the resistance to the process of cryopreservation is more with hematopoietic progenitor cells than other mononuclear cells,[25] also reflected by higher postthaw viability when compared to other nucleated cells. Postthaw assessments are also influenced by the processing done on the product such as washing of the cells which could also influence the appropriate cellular assessment of the samples. In our study, no postthaw manipulations were done on the product [Table 1].

Minimal product manipulations were done on the harvested product (plasma volume reduction) so as to reduce the volume of the final product to avoid excessive DMSO exposure. Similarly, cell concentration optimization is also recommended before cryopreservation. Early literature[26] suggested a cell of $<20 \times 10^6$/ml is appropriate to minimize the loss due to cryofreezing. Based on the recent[27] literature, concentration up to 100×10^6/ml in the product to be freezed is considered acceptable. In our study, we did not conduct a cell correction before cryopreservation, and the mean absolute neutrophil count was $71.1 \pm 47.5 \times 10^6$/ml in the product before cryopreservation [Table 3].

Postthaw infusion, of the cryopreserved product, is reported to cause certain adverse reactions due to the presence of cellular debris and DMSO (causes cellular injury and osmotic imbalance in the recipient). In our analyses, there were very few adverse reactions reported with infusion. Majorly they were milder reactions, except one severe anaphylactic reaction reported in one of the recipients. This is in accordance with earlier reports,[12] which indicate that a lower concentration of DMSO is associated with lesser adverse reactions to the infusion. Table 1 discusses various published studies on cryopreservation using −80°C mechanical freezers with the efficiency and outcome of UCF method for cryopreservation. Our analysis was able to show comparable results in terms of efficiency and posttransplant hematopoietic engraftment.

UCF of stem cells is an effective and useful method to store these cells for long term (<6 months) when compared to CRF. CRF with its added economic burden, requirement of trained manpower, and with not many cryopreservation procedures done across

resource-constrained centers, UCF is a more feasible and economic alternative.

Conclusion

Our retrospective analysis indicates that UCF of hematopoietic stem cells using mechanical freezers at −80°C can be successfully done for patients undergoing autologous transplants with good outcomes. UCF offers a simple, safe, and cost-effective mode of short-term cryopreservation which can be easily adapted in resource-constrained settings.

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Conflicts of interest
There are no conflicts of interest.

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