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Preservative methods for autologous peripheral blood stem cells collected from Thai patients with multiple myeloma

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ABSTRACT

Background: Bone marrow transplantation in multiple myeloma patients is one of the methods for multiple myeloma therapy. Blood stem cell preservation is very important for transplant therapy. Thus, preservative methods of peripheral blood stem cells (PBSCs) must be evaluated for successful transplantation.

Objectives: The aim of this study was to collect and preserve autologous peripheral PBSCs ($CD34^+/CD38^-$) from multiple myeloma patients at 4°C and deep-freezing.

Materials and methods: PBSCs were collected by leukapheresis before being cryopreserved and kept in liquid nitrogen. The number of $CD34^+/CD45^{dim}$ cells were investigated and subpopulation $CD34^+/CD38^-$ cells were evaluated by trypan blue exclusion method and 7-AAD by flow cytometry before and after cryopreservation.

Results: The result showed that $CD34^+/CD38^-$ cells constituted 45.08% of total $CD34^+$ cells and 0.56% of total nucleated cells (TNCs). After thawing, $CD34^+/CD38^-$ cell number did not show significant differences when compared to pre-storage. The CFU recovery after cryopreservation and storage at 4°C for 7 days were 93.53±5.83 and 63.77±12.40%, respectively. Storage at 4°C for 7 days showed significant decrease when compared to day 1. The remaining of total CFU after deep-freezing and storage at 4°C confirmed the tolerant and robust recovery of $CD34^+/CD38^-$ cells. The engraftments of deep-freezing cells were 100% successful within 11 days without graft failure.

Conclusion: In this present study, we assess the process of storage for high quality and recovery of PBSCs at 4°C within 3 days and cryopreservation for development of autologous hematopoietic stem cell (HSC) transplantation in multiple myeloma patients. Moreover, these conditions are important data guideline for HSC preservations and applications in the future.

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Introduction

Multiple myeloma (MM) is a malignancy of bone marrow, caused by defect or dysfunction of the B-cell in various origin of the body. The major characteristics of MM represent the hyperproliferation of plasma cells in bone marrow and found abnormalities of monoclonal protein or M-protein in serum and/or urine may result in end-organ dysfunction and patient died later. The incidence of the disease over the world approximately 4 cases in population of 100,000 persons and a total of 11,300 deaths per year.[1,2] In 2012, multiple myeloma, there are 386 cases in male, accounting for 1 per 100,000 population and 379 cases in female, accounting for 0.8 per 100,000 population and the incidence is between 0.5-2 cases of 100,000 people in Thailand.[2]

Autologous hematopoietic stem cell transplant (autologous-HSCT) has been developed for treatments of multiple myeloma,[3-5] by destroying the abnormal stem cells and replacing with normal patient's hematopoietic stem cells (HSCs). Currently, more advanced treatment regimens have been developed, especially hematopoietic stem cell transplant (HSCT) treatments. Autologous hematopoietic stem cell transplant (autologous-HSCT) and allogeneic hematopoietic stem cell transplant (allogeneic HSCT) are commonly used in routine treatment. Mobilization of autologous-HSCT is performed by chemotherapeutic treatments and/or combined with G-CSF to destroy the cancer or leukemic cells and stimulate HSCs releasing from bone marrow into the bloodstream as peripheral blood stem cells (PBSCs) and collected by leukapheresis. PBSCs were cryopreserved with dimethyl sulfoxide (DMSO) at concentration of 10% v/v is optimal and a common reagent for freezing solution[6] before storage in deep freezing while pretransplant and later thaws before reinfusion to patient for transplantation. PBSCs quality assessment depends on target $CD34^+$ cell concentration, viability, functional, and recovery. The International Society of Hematotherapy and Graft Engineering (ISHAGE) recommend to use HSCs at the concentration of 2-5x10^6 cells/kg body weight (BW) for the successful of HSCT,[7] $CD34^+$ cell enumeration wildly used single-platform ISHAGE protocol measured by flow cytometer to detect $CD34^+/CD45^+$ cells and viability with 7-aminoactinomycin D (7-AAD). The HSC or $CD34^+$ cell measurement by flow cytometry was required the ISHAGE[7] and ISCT & EBMT[8] guidelines for PBSCs, total nucleated cell (TNC) count, $CD34^+$ cell count, cell viability, and % recovery. The measurements of cell viability before and after storages affect $CD34^+$ cell viability.[9,10] Several studies have been found that, the cell viability, ability, and engraftment potential were decreased after cryopreserved PBSC thawing.[11-14] Moreover, it was reported that PBSCs can be stored at 4°C for more than 48 h without significantly decreased in $CD34^+$ cell number and viability.[15-17]

This study aims to assess the preservation methods of PBSCs before and after storage deep freezing cryopreservation and storage at 4°C. The investigation and quantification of $CD34^+/CD45^{dim}$ and subpopulation $CD34^+/CD38^-$ in PBSCs were measured by flow cytometry. Cell viability was performed by trypan blue exclusion method and flow cytometry (7-AAD). Assessed the potency of $CD34^+$ cells by CFU assay in a semi-solid medium to compare the number of total colony-forming unit (CFU) and colony type.[18-20] In this study, we assess the quality of PBSCs and the process of storage methods for the development of autologous-HSCT treatment in multiple myeloma.

Materials and methods

Patients

Seven collections of PBSCs by leukapheresis were obtained from 4 patients (1 male and 3 females) with multiple myeloma who were receiving autologous-HSCT (Table 1). Four multiple myeloma patients (less than 65 years old) who achieved at least very good partial response after treatments was the inclusion criteria. The stem cell mobilization was done with G-CSF alone (10 µg/kg/day) for 5 consecutive days before stem cell collection. All multiple myeloma patients were collected by random sampling during December 2017 to September 2018 at the Maharaj Nakorn Chiang Mai hospital, Chiang Mai province, Thailand. This study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The study code was NONE-2559-04138/Research ID: 4138, Date of Approval: September 23, 2016. The optimal stem cell dose was more than 2x10^6 cells/kg BW. The stem cell collection was done in 1-2 days to achieve optimal stem cell dose. PBSCs were assessed pre and post cryopreservation within 1-6 months after storage. Analysis of PBSCs including volume (mL), TNC count (x10^{10} cells), % recovery, $CD34^+$ cell count (x10^6 cells/kg BW), $CD34^+/CD38^-$ cell count (x10^6 cells/kg BW), and % cell viability. The potency of $CD34^+$ cells were measured by CFU assay. Number of each colony type was counted (x10^4 cells) and calculated into percentage (%). Cell number and viability of PBSCs were compared and analysed of each bag before and after storage in two conditions.

Table 1 Characteristics of autologous PBSCs (n=7) from 4 autologous-HSCT patients.

Parameter	Male	Female
Age* (year)	52.00 (29-60)	
Weight* (kg)	56.00 (41–80)	
Sex	1 (25%)	3 (75%)
Total PBSCs (7 bags)	1 (14.29%)	6 (85.71%)

**Data present as median (range).*

PBSCs collection, aliquot, storage at 4°C, deep freezing, and thawing

Autologous PBSCs (7 blood collection bags) from multiple myeloma patients (n=4) were collected by leukapheresis (COMTEC, Fresenius, Waltham, MA), target $CD34^+$ yield in PBSCs was ≥ 2.0x10^6 cells/kg BW of patient. PBSC bags were centrifuged at 2,000xg, 8 min at 22°C (Sorvall RC3, Thermo scientific, CA, USA) to concentrate TNC and reduce plasma approximately 20%. PBSC samples were immediately aliquoted from pre- and post-centrifugation of PBSCs into cryotubes (1 mL/tube) and measured for TNC and TNC recovery (%). Post-centrifuged PBSCs were aliquoted in two vials for pre-storage determination and CFU assay.

Remaining post-centrifuged PBSCs were diluted with cryopreservative solution with the ratio of 1:1 in cryobag. Cryopreservative solution was prepared by mixing DMSO and plasma with the final concentration of DMSO adjusted to 20% and then stored at 4°C for 15 min. The final concentration of DMSO after adding PBSCs was 10%. Cryopreserved PBSCs were aliquoted in two cryotubes (1 mL in each) for post-thawing determination and CFU assay. Then, cryopreserved PBSCs were stored into pre-frozen cassette at -20°C for 15 min and further deep freezing by controlled-rate freezer to -90°C. Finally, all samples were stored in a liquid nitrogen tank (-180°C). Post-thawing samples were thawed at the indicated time period at 37°C in water bath for 2-3 min. Samples were aliquoted into two tubes; the first tube was resuspended with EDTA at the sample to EDTA ratio of 500:50 for a viable TNC count by trypan blue exclusion method and flow cytometry analysis using 7-AAD. The second tube was resuspended with heparin at the sample to heparin ratio of 500:80 for the CFU assay. Moreover, 7 PBSC bags were aliquoted approximately 5 mL into sample bag and stored at 4°C for 7 days. Cold storage sample bags were aliquoted in two cryotubes. The first cryotube was measured for $CD34^+$ and $CD34^+/CD38^-$ cell counts by flow cytometry. TNC and cell viability were determined by trypan blue exclusion method and 7-AAD. The second cryotube was assayed for CFU at day 1, 3, 5, and 7.

TNC, $CD34^+/CD45^{dim}$, and $CD34^+/CD38^-$ cell counts

TNCs were counted using a cell counter (XE-5000, Sysmex, Kobe, Japan). HSC analysis were performed by using a modified ISHAGE protocol[7]. HSCs ($CD34^+/CD38^-$) were determined by stem cell enumeration kit (BD Bioscience, CA, USA). Briefly, samples were diluted to $5x10^5$ cells/100 µL with 0.5% BSA in PBS, pH 7.4. Sample (100 µL) was incubated with CD45-FITC/CD34-PE antibodies for $CD34^+$ cell or HSC identification and anti-CD38 antibody (CD-38-PECy7) for subpopulation $CD34^+/CD38^-$ identification in fluorescent bead tube (TruCount, BD Bioscience, CA, USA) followed by 20 min incubation at room temperature in the dark. After that, 2 mL red blood cell lysis buffer (ammonium chloride, BD Bioscience, USA) was added and followed by incubation for 10 min in the dark and analysis within 1 h. HSCs were enumerated using a single platform analysis on a Cytomics FC500 flow cytometer (Beckman Coulter, IN, USA) to identify target $CD34^+$ cell ($CD34^+/CD45^{dim}$, SS^{low}) and subpopulation of HSCs ($CD34^+/CD38^-$).

$CD34^+$ enumeration was completed by using known a fluorescent bead tube, collecting 75,000 of $CD45^+$ events in each sample. Calculation of $CD34^+$ or $CD34^+/CD38^-$ cells/µL = (number of $CD34^+$ or $CD34^+/CD38^-$ events/number of beads counted)x(total number of beads in each bead-coated tube) x(dilution factor/sample volume).

Cell viability test by trypan blue exclusion method

PBSCs were tested for their viability by trypan blue exclusion method. Samples were diluted with PBS, pH 7.4 with the ratio of 1:10 and then mixed with an equal volume of trypan blue vital stain (0.4%) at room temperature for 5 min. The dead cells were identified by a blue colour of cytoplasm (stained cells as dead cells cannot exclude trypan blue dye). Stained and unstained cells were counted under 10X light microscope (Olympus, USA) and calculated % viability = unstained cells/(unstained cells+stained cells)x100.

Cell viability test by flow cytometry

Samples were stained with 7-aminoactinomycin D (7-AAD) using stem cell enumeration kit (BD Bioscience, CA, USA) and analysed by FC500 flow cytometer (Beckman Coulter, IN, USA). Nucleus was stained with fluorescent dye 7-AAD, and used to detect the signal of viable cells by flow cytometry. This study measured the cell count and cell viability by diluting $5x10^5$ cells/100 µL with 0.5% BSA in PBS, pH 7.4. A BD-Trucount tube contained fluorescent bead that used to calculate the absolute cell count was used. Samples (100 µL) were incubated with 7-AAD (5 µL) in BD-Trucount tube for 20 min at room temperature in the dark. Next, 2 mL of lysing solution (1X ammonium chloride (NH_4Cl) solution, BD Bioscience, USA) was added into the tube, mixed by vortexing and incubated in the dark for 10 min. Samples were not fixed or washed before analysis. Finally, sample tubes were placed in ice bath and immediately analysed within 1 h by flow cytometer (Cytomics FC500, Beckman, USA). The results were analysed by Flowjo analysis software based on ISHAGE guidelines.

CFU assay

Colony-forming unit was used for the indirect assessment of viability as well as potency of PBSCs. The CFU assay represents the clonogenic potential of HSC differentiation to mature cells from viable progenitor cell lineages. Red blood cells in PBSCs were lysed by NH_4Cl solution with the ratio of 1:4 followed by suspended and washed 3 times with 2% FBS in IMDM (Invitrogen™, Carlsbad, CA, USA). A final cell suspension ($1-5x10^4$ cells) was resuspended in methylcellulose medium (HSC003, R&D system, MN, USA) supplemented with cytokine cocktails (50 ng/mL SCF, 10 ng/mL GM-CSF, 10 ng/mL IL3, 3 IU/mL EPO) and plated in 35 mm cell culture dish in triplicate following the manufacturer's instruction. The colony types from progenitors were identified and counted, including CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-E, and BFU-E. Total colony count ($x10^4$ cells) and % CFU type were scored.

Statistical analysis

All results were expressed as mean±SD with significant difference level of $p<0.05$ and the differences were analysed by ANOVA and t-test.

Results

Effects of deep-freezing in cryopreservation and storage at 4^0C on $CD34^+$, $CD34^+/CD38^-$ cells, and TNC count by flow cytometry

$CD34^+$ cells were collected from multiple myeloma patients, acquiring an average number of $12.14±19.62x10^6$ cells/kg BW. $CD34^+$ cell population in the TNCs after leukapheresis was 3.03%. $CD34^+$ cell lost after deep-freezing was 3.29%. Pre-storage $CD34^+/CD38^-$ cells were $7.78±13.59x10^6$ cells/kg BW. Thus, $CD34^+/CD38^-$ cell numbers were 62.74% of total $CD34^+$ cells and 1.94% of TNCs. After thawing, $CD34^+/CD38^-$

cells (7.61±13.36x10^6 cells/kg BW) constituting 64.82% of total $CD34^+$ cells and 2.11% of TNCs were not significantly different (p>0.05) when compared to those of pre-storage. After 1-6 months (average 3.57±2.43 months) of cryopreservation, the number of $CD34^+$ and $CD34^+/CD38^-$ cells showed no significant difference when compared to pre-storage (p>0.05) data. However, TNCs showed significant differences after deep freezing (p<0.05) with total cell loss of 9.98% (Table 2).

$CD34^+$ cells after storage at 4°C for 3, 5, and 7 days decreased viable cell numbers by 15.6, 38.12, and 67.09%, respectively when compared to day 1. Moreover, $CD34^+/CD38^-$ cells decreased by 17.16, 30.08, and 58.20%, respectively when compared to day 1 (Table 3). However, there was no significant difference of both $CD34^+$ and $CD34^+/CD38^-$ cell number after storage at 4°C for 3, 5, and 7 days when compared to day 1.

Table 2 $CD34^+$ stem cells, $CD34^+/CD38^-$ cells, and TNC count from pre-storage (post-centrifugation) and post-thawing cryopreserved PBSCs.

Parameter	Pre-storage	Post-thawing	*p* value
$CD34^+$ cells (x10^6 cells/kg)	12.14±19.62	11.74±19.33	>0.05
$CD34^+$ cell loss (x10^6 cells/kg)	0.39±0.51		
$CD34^+/CD38^-$ cells (x10^6 cells/kg)	7.78±13.59	7.61±13.36	>0.05
$CD34^+/CD38^-$ cell loss (x10^6 cells/kg)	0.16±0.22		
TNC count (x10^8 cells/kg)	4.01±1.88	3.61±2.32*	<0.05
TNC count loss (x10^8 cells/kg)	0.62±0.67		
% TNC recovery	85.99±28.59		
Volume of PBSC bag (mL)	173.29±64.96	40.29±11.55*	<0.05

*All data are shown as mean±SD with significant difference *p<0.05 when compared to the pre-storage, n=7.*

Table 3 $CD34^+$ stem cells, $CD34^+/CD38^-$ cells, and TNC count of PBSCs storage at 4°C for 1-7 days.

Parameter	Day			
	1	3	5	7
$CD34^+$ cells (x10^6/kg)	12.14±19.62	11.19±18.05	9.15±14.62	5.53±8.72
$CD34^+$ cells (%)	100	85.40±10.89	61.88±23.39	32.91±18.54
$CD34^+/CD38^-$ cell (x10^6/ kg)	7.78±13.59	7.11±12.25	5.72±9.71	3.80±6.49
$CD34^+/CD38^-$ cell (%)	100	82.84±12.96	69.92±10.10	41.80±19.71
TNC count (x10^8/kg)	4.01±1.88	3.86±1.91	3.68±1.90	3.49±1.88

All data are shown as mean±SD, n=7.

Percentage of cell viability after deep-freezing in cryopreservation and storage at 4°C by trypan blue exclusion method and 7-AAD by flow cytometry

Cell viability after deep-freezing in liquid nitrogen were determined by trypan blue exclusion method and compared to those of 7-AAD by flow cytometer. Pre-storage and post-thawing cell viabilities were also determined and compared. The results showed that viable cells of pre-storage were 96.78±1.45 and 97.96±0.73% by trypan blue and 7-AAD, respectively (Table 4). After thawing, the viable cells were 64.80±6.19 and 70.43±13.48% when determined by trypan blue exclusion method and flow cytometry, respectively (Table 4). The percentage of cell viabilities were significantly decreased by 33.04 and 28.10%, respectively when compared to the pre-storage (p<0.05). Cell viability after storage at 4°C was determined by trypan blue exclusion method and compared to those of 7-AAD for 3, 5, and 7 days. The viable cells decreased by a time-dependent manner when determined by both methods. The percentages of cell viability at day 7 were significantly decreased by 17.71 and 14.26%, respectively by trypan blue exclusion method and flow cytometer, respectively when compared to day 1 (p<0.05). Moreover, the percentage of cell viability at day 5 was significantly decreased by 12.42% by trypan blue exclusion method when compared to day 1 (p<0.05) (Table 5).

Table 4 Percentage of cell viability after deep freezing and determining by trypan blue exclusion method and 7-AAD by flow cytometry.

Cell viability	Pre-storage	Post-thawing	*p* value
Trypan blue exclusion (%)	96.78±1.45	64.80±6.19*	<0.05
Flow cytometry (7-AAD) (%)	97.96±0.73	70.43±13.48*	<0.05

*All data are shown as mean±SD with significant difference,*p<0.05, when compared to the pre-storage, n=7.*

Table 5 Percentage of cell viability by trypan blue exclusion and flow cytometry (7-AAD) of PBSCs storage at 4 ^{O}C for 1-7 days (n=7).

Day	1	3	5	7
Trypan blue exclusion (%)	97.34±0.40	94.70±2.19	87.58±4.55*	79.63±7.44*
Flow cytometry (7-AAD) (%)	98.37±0.73	96.36±2.10	92.36±3.12	84.11±7.61*

*All data are shown as Mean±SD with significant p<0.05. *Result was significant difference when compared to day 1.*

Effects of deep-freezing cryopreserved and storage at 4°C on colony forming unit (CFU) of progenitor and committed cell growths

We next evaluated whether deep-freezing cryopreserved $CD34^+$ cells in liquid nitrogen had the ability to grow in the methylcellulose cultures with cytokine cocktails. $CD34^+$ cells gave rise to various types of myeloid colonies including CFU-granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM), CFU-granulocyte/monocyte (CFU-GM), CFU-granulocyte (CFU-G), CFU-monocyte (CFU-M), burst-forming unit erythroid (BFU-E), and CFU-erythroid (CFU-E). BFU-E showed the highest colony counts in both pre-storage and post-thawing, followed by CFU-G and CFU-GM, respectively. Colony counts of post-thawing showed no significant difference when compared to the pre-storage as shown in Table 6.

After PBSCs were kept at 4°C for 3, 5, and 7 days. The CFUs were determined and identified all colonies. Total colony number after storage for 3, 5, and 7 days decreased by 3.90, 10.39, and 27.27%, respectively when compared to the day 1. CFU recovery (%) at day 5 was also significantly decreased that indicated in Table 7. However, CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E counts of storages in day 3, 5, and 7 were not significant difference when compared to the day 1 as shown in Table 7.

Table 6 Colony forming unit (CFU) counts of progenitor and committed cell growths before deep-freezing and after thawing from cryopreservation.

CFU	Pre-storage (%)	Post-thawing (%)	*p* value
CFU-GEMM	1.14±0.72	1.10±0.59	>0.05
CFU-GM	10.75±5.11	4.81±5.66	>0.05
CFU-G	26.48±15.89	26.59±25.40	>0.05
CFU-M	1.00±0.75	0.95±0.73	>0.05
CFU-E	0.48±0.38	0.57±0.69	>0.05
BFU-E	38.76±17.44	46.48±6.41	>0.05
Total (colonies/1.0x10^6 cells)	153.45±208.57	139.83±189.96	0.005
% CFU recovery	93.53±5.83		

All data are shown as mean±SD, n=7.

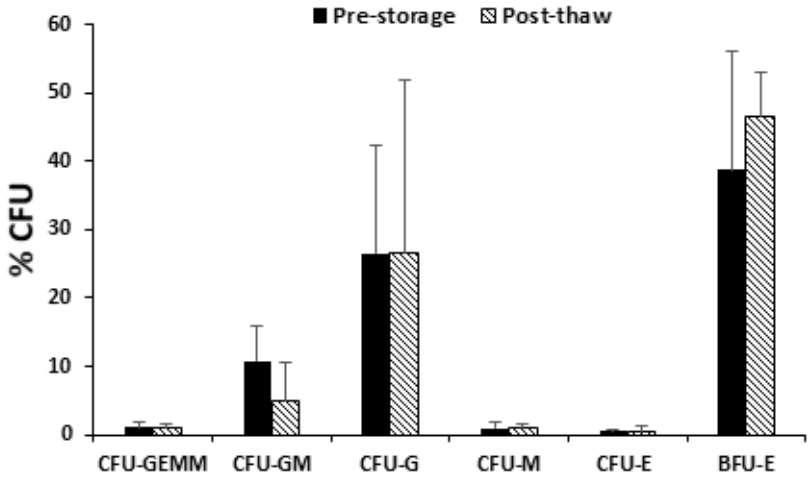

Figure 1. *Colony forming unit (CFU) counts of progenitor and committed cell growths before deep-freezing and after thawing from cryopreservation.*

Table 7 CFU counts and identification types of PBSCs storage at 4^{O}C for 7 days.

Colony forming unit	Day			
	1	3	5	7
CFU-GEMM (%)	1.23±0.99	1.24±0.52	1.15±0.44	0.76±0.69
CFU-GM (%)	9.27±4.74	10.95±3.13	9.57±6.93	8.69±7.17
CFU-G (%)	58.67±14.89	52.01±0.17	53.27±11.21	52.19.11±9.71
CFU-M (%)	0.76±0.73	0.75±0.72	1.00±0.76	0.52±0.50
CFU-E (%)	0.61±0.90	0.19±0.50	0.27±0.49	0.32±0.61
BFU-E (%)	44.27±7.80	40.44±7.11	41.07±4.29	44.09±6.26
Total (colonies/1x10^{6} cells)	1.54±2.07	1.48±2.00	138.15±192.32	112.43±161.59
%CFU recovery	100.00	93.93±3.14	83.39±7.24*	63.77±12.40*

*All data are shown as mean±SD with significant different *p<0.05 when compared to the pre-storage at day 1, n=7.*

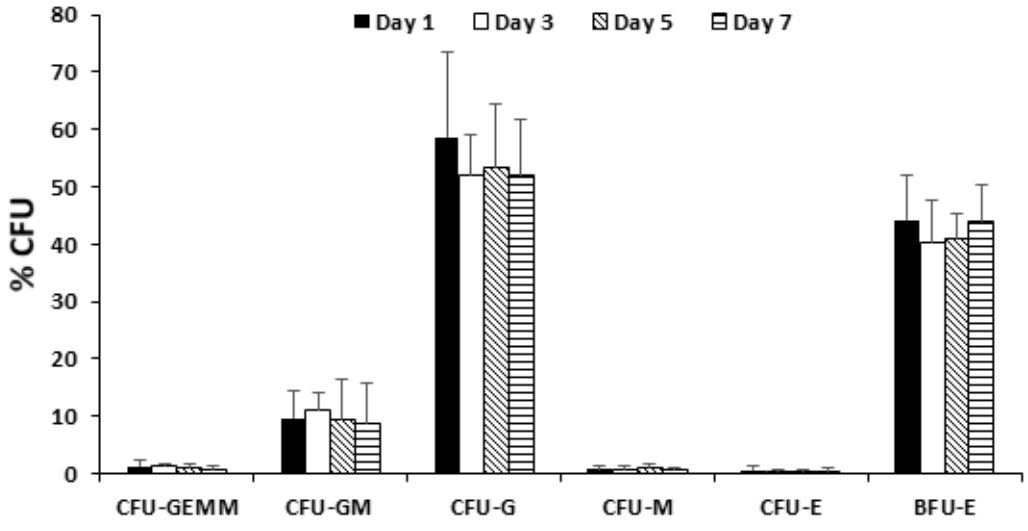

Figure 2. *CFU counts and identification types of PBSCs storage at 4^{O}C for 7 days.*

Evaluation of storage PBSCs by deep-freezing on clinical transplantation and engraftment in multiple myeloma patients

This study, storage PBSCs by deep freezing were transplanted to multiple myeloma patients. A day after stem cell collection, patients underwent autologous-HSCT with melphalan 200 mg/m^2 in 2 consecutive days. On the next day, non-cryopreserved stem cell was transfused. Thus, PBSCs were stored for 3 days after collection. To evaluate the engraftment, HSCs from 7 samples were transplanted to the multiple myeloma patients. All bags from leukapheresis procedures were infused autologously in each own patient (100%). The total CD34$^+$ cells were 12.14±19.62x10^6 cells/kg BW infused (CD34$^+$/CD38$^-$ cell number was ~11.74x10^6 cells/kg BW). After infusion, absolute neutrophil count (ANC) and platelets were engrafted within 11.25±0.96 and 11.50±1.00 days (Table 8). ANC was more than 0.5x10^9 cells/L and platelet counts were more than 20x10^9/L in 3 consecutive days without transfusion support. There were no graft failures in any cases.

Table 8 Clinical transplantation and engraftment in multiple myeloma patients after deep-freezing.

Leukapheresis procedures (Bag)	Total CD34$^+$ cells (x10^6/kg infused)	PBSC infused (Bag)	Engraftment	
			ANC (Day)	Platelet (Day)
1.75±0.96	12.14±19.62	2.00±1.15	11.25±0.96	11.50±1.00

All data are shown as mean±SD, n=7.

Discussion

Storage HSCs is very important for bone marrow transplantation. HSC preservation method are based on reasonableness of hospitals and doctors. Thus, both deep-freezing and storage at 4^{O}C have been used for bone marrow transplantation of multiple myeloma. Deep-freezing cryopreservation is the conventional method for hematopoietic stem cell (HSC) storage; it is a critical part of hematopoietic stem cell transplantation. Leukapheresis has been used to collect the peripheral blood stem cells (PBSCs) from multiple myeloma patients and transplant to those patients who have been recommended to replace their bone marrow with their own HSCs (autologous-HSCT). Cellular therapy products such as PBSCs may need to be cryopreserved until their use. There are two major methods of cell cryopreservation: deep-freezing and storage in liquid nitrogen at < -180^{O}C and freezing at -80^{O}C. Deep-freezing method is recommended for long term storage for years while freezing at -80^{O}C lacks evidence for its safety. However, freezing at -80^{O}C was reported to be used for storage in a region with limited resources.[21] The recovery of viable CD34$^+$ cell populations after deep-freezing was 91%. This result shows the same pattern of previous reports where the recovery of nucleated cells and viable CD34$^+$ cells for adult (n=51) stem cell collections were 92 and 91%, respectively.[22,23] CD34$^+$ cells were commonly reported on cell viability. However, CD34$^+$ is a common marker of both HSCs and progenitors in bone marrow. There is no report that the cell viability and engraftment of CD34$^+$/CD38$^-$ cells after deep-freezing and thawing. In this study, subpopulation of CD34$^+$/CD38$^-$ cells (HSCs) was focused and observed for total cell number of HSCs in PBSCs before and after deep-freezing cryopreservation by flow cytometry. Thus, this is a first report to specify the viability of CD34$^+$/CD38$^-$ cells after preservation. CD34$^+$/CD38$^-$ cell number after collection was 12.14±19.62x10^6 cells/kg BW, 3.03% of total CD34$^+$ cells, and 1.94% of TNCs. After thawing CD34$^+$/CD38$^-$ cell number did not show significant difference when compared to that of pre-storage (p>0.05). CD34$^+$/CD38$^-$ cell loss was 2.18%. Storage at 4^{O}C is a routine method used for multiple myeloma patients at the Maharaj Nakorn Chiang Mai hospital, Chiang Mai province, Thailand. CD34$^+$/CD38$^-$ cells after storage at 4^{O}C for 3, 5, and 7 days were decreased by a time-dependent manner. At day 7 showed significant decrease percentage of cell viabilities in both trypan blue exclusion method and 7-AAD assay. These results related to percentage of CFU recovery (Table 7). When CFU types were observed, there were no significant different between day 3, 5, and 7 as compared to day 1. The viability of collected PBSCs storage at 4^{O}C was significantly difference from day 1 (data from Table 5) therefore it might be suitable for transplant up to 3 days with cell viability of 94.70±2.19% by trypan blue exclusion method. If cell viability was determined by 7-AAD assay, the values would show the cell viability higher than that of trypan blue exclusion method at the same day. These two methods are popular for cell viability determination. Trypan blue exclusion method is the conventional method to show a loss of cell permeability that presents unsuitable cells for further application in patients. Cell viability of CD34$^+$ cells after 7-AAD assay was previously reported with values ranging from 58.50-99.48%.[24,25] However, the results after transplantations showed the good engraftment of HSCT. PBSCs were reported to store at 4^{O}C for more than 48 h without significantly decreased in CD34$^+$ cell number and viability.[15-17] Moreover, cell viability after deep freezing which (performed by trypan blue exclusion and flow cytometry) showed significant difference when compared to the pre-storage (p<0.05) by the values of 33.04 and 28.10%, respectively. This result suggested that other nucleated cells (not including the CD34$^+$ or CD34$^+$/CD38$^-$ cells) were dead after thawing. This phenomenon was observed in TNCs after thawing that were significantly decreased (p<0.05) by 9.98% when compared to the pre-storage. CD34$^+$/CD38$^-$ cells and CD34$^+$ (HSCs and progenitor cells) cells seem to be well adaptable compared to other nucleated cells.[26] CD34$^+$ cells tolerate up to 60 min exposure to 25% w/w (3.2 M) DMSO at +2^{O}C with no significant loss in clonogenic capacity.[6] Different cell types tolerated various ranges of solution osmolarities that effect by osmotic shocks, which

was evidenced by an increased necrosis in neutrophils and apoptosis in monocytes. Mature myeloid cells are more sensitive to osmotic stress than lymphocytes and $CD34^+$ cells. Moreover, $CD34^+/CD38^-$ cells appeared more resistant to cryoinjuries than their $CD34^+/CD38^-$ counterpart.[26,27] HSCs from 40 bone marrow and peripheral blood samples were previously reported to be successfully preserved for long-term cryostorage (5-14 years) using a standard in vitro method. Forty percent of harvests had $CD34^+$ cell counts of at least $0.7x10^6$/kg BW and 85% had CFU-GM counts of at least $1.0x10^5$/kg BW.[28] The potency of $CD34^+$ cells was assessed by CFU assay in a semi-solid medium to compare the number of CFUs and colony types.[19,20,29] The result completely showed that all CFU counts of deep freezing after thawing did not significantly differ from cell growth when compared to pre-storage (p>0.05). The percentage of CFU recovery before storage was 93.53±5.83. In this study, we assessed the quality and recovery of PBSCs as a function of storage process for the enhancement of autologous-HSCT treatment in multiple myeloma. The remaining of total CFU after deep-freezing and storage at 4^oC confirmed the tolerant and robust recovery of $CD34^+/CD38^-$ cells. The engraftments of 4^oC and deep-freezing cells were 100% successful within 11 days without graft failure. Absolute neutrophil count (ANC) should present more than $0.5x10^9$ cells/L[30] while platelet count should be more than $20x10^9$ cells/L in 3 consecutive days without transfusion support.[31]

Conclusion

In conclusion, deep freezing for 1-6 months and storage at 4^oC within 3 days are recommended methods for PBSC preservation. These methods provide cell viability and function for bone marrow transplantation with high successful probability in multiple myeloma patients.

Conflicts of interests

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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