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ORIGINAL ARTICLE

Evaluation of a Cryopreservation Procedure to Set Up a New Bone Marrow Transplant Unit Using Lymphocyte Proliferation Test

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A bone marrow transplant (BMT) is one kind of standard treatment modality in advanced hemato-oncology. In order to set up a BMT unit, one of the important steps before starting a clinical program is to evaluate the cryopreservation procedure for stem cell storage. Twenty one bags of buffy coat were used to be the testing specimens. They were processed and frozen according to cryopreservation protocol and kept in liquid nitrogen for 2 weeks. The evaluation process was carried out with a lymphocyte proliferation test together with trypan blue staining. By measuring the optical density of each lymphocyte containing well after stimulation, the lymphocyte proliferation value (LPV) could be obtained. When comparing them before and after cryopreservation, the LPV was 2.064 ± 0.379 (mean ± SD) and 1.913 ± 0.546, ($p = 0.314$), respectively. At 2 weeks after cryopreservation, comparing between the frozen group and the unfrozen control, the LPV was 1.913 ± 0.546 and 0.486 ± 0.453, ($p < 0.05$), respectively. The LPV showed clear efficacy of the procedure, especially for preserving the cellular

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proliferation function. Our model of the cryopreservation procedure evaluation at preclinical phase by use of a buffy coat and lymphocyte proliferation test seems feasible for newly-established small BMT units. With these results, clinical transplantations can be performed with more confidence.

Keywords Cryopreservation, Bone marrow transplantation, Lymphocyte proliferation test.

INTRODUCTION

A bone marrow transplant (BMT) is one standard treatment modality in advanced hemato-oncology. To set up a BMT unit, one of the important steps before starting a clinical program is to evaluate the cryopreservation procedure for stem cell storage. When first establishing BMT units, there are many particular issues to consider for the evaluation of the cryopreservation protocol before starting treatments with real patients. Firstly in general, evaluations of the procedure are usually carried out in parallel with clinical transplantations. Performing quality evaluations by using stem cell products before launching a clinical program is not feasible. Even though new cell processing laboratories require more practice to ensure their processing techniques before manipulating real stem cell products, no technical rehearsals can be performed without suitable testing specimens. Secondly, in routine transplantations, the standard methods for stem cell product evaluations are viable CD34+ enumeration with flow cytometry (Lee, 2008) and clonogenic assay (Coligan, 1996) of hematopoietic progenitor cells. Before these standards can be developed and implemented, alternative means are needed for evaluating the quality of the cryopreservation procedure.

Therefore, to carry out quality evaluations of the procedure without authentic stem cell collection and without standardized evaluation methods yet in place, this study finds buffy coat from healthy blood donors to be sufficiently suitable as testing specimens and for product processing rehearsals. Here is described our model of cryopreservation procedure evaluations before starting a clinical program for a small new BMT unit. This work does not mean to propose a new stem cell evaluation without using flow cytometry and clonogenic assay, but it tries to find out a new method to ensure quality and to evaluate the readiness of cryopreservation procedure without authentic stem cell collection before commencing clinical transplantations.

MATERIALS AND METHODS

For the testing specimens, 21 bags of buffy coat were used. They were collected from healthy blood donors by using quadruple bags with bottom and top system in a routine blood donation (Högman, 1988, 1998). Buffy coats were

processed and frozen according to protocol and kept in liquid nitrogen for 2 weeks. There were two separate samples from the same donor, one of which was kept unprocessed in a 4°C-refrigerator as the control.

Because of no stem cells in buffy coat products, lymphocytes were the most suitable surrogate for testing viability. Therefore, the evaluation process was carried out with a lymphocyte proliferation test together with trypan blue staining. By measuring the optical density of each lymphocyte containing well after stimulation, the lymphocyte proliferation value (LPV) could be obtained and could represent the viable lymphocytes. The LPV and the percentage of viable cells from the trypan blue staining were compared in each product before and 2 weeks after the freezing process. They were also compared between the 2-weeks frozen group and the unfrozen control. The study was approved by the institutional ethics committee. Written informed consent was obtained from all blood donors before entering the study.

Cryopreservation Protocol

In the frozen group after processing, the final products had the nucleated cell concentrations decreased by half of the original buffy coat's concentrations by adding dimethyl sulfoxide (DMSO) and plasma. They were contained in 10% DMSO. Details of nucleated cell concentration and product volume are shown in Table 1. This protocol was designed to be compatible with future apheretic products with similar volumes but with more nucleated cell concentrations. The freezing process was started with a controlled rate freezer and the final temperature of the products reached −80°C before being transferred to a −196°C liquid nitrogen tank. The freezing protocol was commenced with a 4°C waiting period. The cooling process was started at a rate of 1°C per minute until −12°C was reached. Then, the cooling rate was increased to 4°C per minute from −12 to −20°C. Usually, the transition from a liquid to a solid phase would take place during this step and would be demonstrated as minimal and short increments of the products' temperature from fusion heat release. The temperature was sustained at −20°C for 5 minutes and cooling was started again with a rate of 1°C per minute from −20 to −40°C. Then,

Table 1: Concentration of nucleated cells and volume of buffy coat products; before and after product processing.

	N	Before	After
Concentration of cells (x10^6 cell/mL)	21	40.87 ± 13.52	21.28 ± 7.67
Volume (mL)	21	46.41 ± 16.98	76.33 ± 32.11

Values demonstrate as mean ± SD.

the cooling rate was increased to 3°C per minute until the final temperature of −80°C was reached. At the end of the protocol, the frozen products were transferred to a −196°C liquid nitrogen tank.

Lymphocyte Proliferation Test

In the lymphocyte proliferation assay, phytohemagglutinin (PHA) was used to stimulate the lymphocytes in order to assess their capacity to proliferate. Briefly, the lymphocytes were isolated from the buffy coats by centrifugation with Lymphoprep reagent (Axis-Shield PoC AS, Oslo, Norway). The resulting lymphocytes were resuspended in a complete medium, RPMI-1640 medium (JRH Biosciences, Hampshire, USA) supplemented with 2 mM L-glutamine (Biochrom AG, Berlin, Germany), 100 U / mL penicillin, and 100 μg / mL streptomycin (Sigma, Missouri, USA). Then, the cells were counted and adjusted to 1×10^6 cells / mL.

The assays were carried out by using 96-well round bottom tissue culture plates (Thermo Scientific Nunc ™, Roskilde, Denmark). Added to each well were isolated lymphocytes ($1x10^5$ cells), which were re-suspended in a complete medium in the presence of PHA (Sigma) at a final concentration of 10 μg/mL. The lymphocyte cultures were then incubated for 96 hours before the cell proliferation was assessed by using the Cell Proliferation ELISA, BrdU kit (Roche Applied Science, IN, USA), according to the manufacturer's instruction. By measuring the optical density of each lymphocyte containing well after stimulation, the LPV was obtained, which represented the viable lymphocytes. Previous lymphocyte proliferation assays using BrdU have been well reported (Kayser, 1996; Hawker, 2003; Pongcharoen, 2007).

Statistical Analysis

A paired *t*-test was performed to compare the mean value of the LPV and the percentage of viable cells from trypan blue staining before and 2 weeks after the freezing process. These values were also compared between the 2-weeks frozen group and the unfrozen control. A P-value of less than 0.05 was set as a level of significance.

RESULTS

With 21 samples, the LPV and the percentage of viable cells from trypan blue staining were used for comparison in each sample before and 2 weeks after cryopreservation. They were also compared between the 2-weeks frozen group and the unfrozen control as shown in Table 2.

Table 2: Lymphocyte proliferation value (LPV) and percentage of viable cells from trypan blue staining; before and after cryopreservation, frozen and unfrozen.

	Pre-freeze	**Post-freeze**	**P-value**
LPV (optical density)	2.064 ± 0.379	1.913 ± 0.546	0.314
Trypan blue staining (%)	97.46 ± 5.20	86.37 ± 7.46	<0.05
	Frozen	**Unfrozen**	**P-value**
LPV (optical density)	1.913 ± 0.546	0.486 ± 0.453	<0.05
Trypan blue staining (%)	86.37 ± 7.46	78.30 ± 10.94	<0.05

Values demonstrate as mean ± SD.

DISCUSSION

The LPV showed clear efficacy of the cryopreservation procedure, especially for the function of preserving cellular proliferation. With LPV, lymphocyte proliferation tests are able to demonstrate a quantitative evaluation of product viability. After cryopreservation, the frozen products had a 92.7% pre-freeze value, in contrast with the result of 23.5% from the unfrozen control. Accordingly, the trypan blue staining also demonstrated protocol efficacy above the control, with a certain amount of cell death in the freezing process. However, as compared with the LPV, the percentage of viable cells from the trypan blue staining of the unfrozen group still remained close to pre-freeze value (78.30 ± 10.94 and 97.46 ± 5.20, respectively), implying trypan blue sensitivity. Even though the intact cellular pumping capacity could be demonstrated by the trypan blue wash out, the cellular proliferative function already declined. However, since evidence has not yet been established about the correlation between LPV and viable CD34+ or colony-forming unit recovery after cryopreservation, this data only shows that our cryopreservation protocol enables to maintain the viability and functionality of the lymphocytes.

Evaluations of the cryopreserved products based on immunophenotype and clonogenic assay of the hematopoietic progenitor cells for clinical hematopoietic stem cell transplantation (HSCT) have been well described (Alencar, 2010; Balint, 1999). However, no reports have documented this issue for newly established BMT units before commencing clinical programs. This product evaluation approach was initially intended for pre-clinical usage during the establishment of a BMT unit without authentic stem cell product collection. More challenges may be addressed about whether or not lymphocyte proliferation tests would be reasonable for evaluating the quality of hematopoietic

stem cell products in the clinical HSCT. With CD34+ enumeration for stem cell adequacy evaluation and lymphocyte proliferation tests for product quality evaluation, together these simple evaluation tests may also prove to provide a valuable evaluation model for clinical HSCT in small BMT units.

Unlike cord blood with plenty of red blood cells, product contents in buffy coats, which have plenty of nucleated white blood cells but no stem cells, tend to more resemble apheretic products in routine peripheral blood stem cell transplantations. Furthermore, because of the lesser nucleated cell concentrations, cord blood needs more special volume preparation before cryopreservation. Therefore, buffy coat products seem more applicable for future routine peripheral blood stem cell (PBSC) collections, and they are also more feasible for product evaluations.

Our model of the cryopreservation procedure evaluation at the pre-clinical phase by use of a buffy coat and lymphocyte proliferation test seems feasible for newly-established small BMT units. With these evaluation results, clinical HSCT programs can be carried out with more confidence. This work describes our experience of the cryopreservation procedure evaluation before commencing a clinical HSCT in a 160-bed university hospital located outside of Bangkok. Our institute has now successfully performed 7 transplantations in patients with hematological malignancies by using autologous PBSC.

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