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Novel cryoprotectant significantly improves the post-thaw recovery and quality of HSC from CB

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Background

Hematopoietic stem cells (HSC) have traditionally been frozen using the cryoprotectant DMSO in dextran-40, saline or albumin. However, the process of freezing and thawing results in loss of HSC numbers and/or function.

Methods

This study investigated the use of CryoStor™ for the freezing of HSC from cord blood (CB). CB donations (n = 30) were collected under an Institutional Ethics Committee-approved protocol, volume reduced and frozen using three different methods of cryoprotection. Aliquots were frozen with either 10% DMSO in dextran-40, 10% DMSO in CryoStor™ or 5% DMSO in CryoStor™. Prior to freezing samples were separated for nucleated cell (NC) and CD34$^+$ counts and assessment of CD34$^+$ viability. Aliquots were frozen and kept in vapor phase nitrogen for a minimum of 72 h. Vials were rapidly thawed at 37°C and tested for NC and CD34$^+$ counts and CD34$^+$ viability and colony-forming unit (CFU) assay.

Results

Cells frozen with CryoStor™ in 10% DMSO had significantly improved NC ($P < 0.001$), CD34$^+$ recovery, viable CD34$^+$ ($P < 0.001$) and CFU numbers ($P < 0.001$) compared with dextran in 10% DMSO. CryoStor™ in 5% DMSO resulted in significantly improved NC ($P < 0.001$) and CFU ($P < 0.001$).

Discussion

These results suggest that improved HSC recovery, viability and functionality can be obtained using CryoStor™ with 10% DMSO and that similar if not better numbers can be obtained with 5% DMSO compared with dextran-40 with 10% DMSO.

Keywords

cryoprotectant, recovery, viability.

Introduction

Cord blood (CB) is a rich source of hematopoietic stem cells (HSC) and has been used in the treatment of children and adults with a range of hematologic, genetic and malignant disorders. Nucleated cell (NC) content of a CB donation is approximately 1 log less than that of a BM donation, and consequently cell dose for transplant is lower. There is a direct correlation between cell dose and both speed of engraftment and survival [1–3]. Maintaining the integrity of the stem cells, from the time of collection, through processing and freezing, to when they are administered to the patient, is critical.

HSC are routinely cryopreserved in nitrogen at temperatures of −196°C; at these low temperatures almost all biologic functions are halted. The blood is kept at this temperature until it is ready to be used; this process involves rapidly thawing the blood in a waterbath at 37°C before being administered to the patient. The processes that cause the most damage to the cells are the freezing and thawing. In order to reduce the damaging effect of cryopreservation, DMSO is used. DMSO is an intracellular cryoprotectant that displaces water from within the cell and thus reduces the formation of ice crystals that would otherwise damage the cell. DMSO, however, is toxic to cells when exposed to temperatures above freezing. DMSO has also been associated with adverse effects to the transplant recipient.

HSC have been traditionally frozen using DMSO as cryoprotectant, diluted in dextran-40, saline or albumin, among others. Concentrations of 10% are widely used and there are also data showing 5% to be equally effective [4]. Worldwide, CB banks have been using cryopreservation protocols validated for other hematopoietic blood products [5] and validated for use with CB. However, there is a loss

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of cells put through the freeze–thaw cycle and new approaches that minimize this effect would be a step forward.

The product CryoStor™ (Biolife Solutions, Owego, NY, USA), is composed of dextran-40, sodium, potassium, calcium, magnesium, phosphate, HEPES, lactobionate, sucrose, mannitol, glucose, adenosine and glutathione. It has a pH of 7.6 and an osmolarity of 360. It is protein and serum free. The manufacturers have evaluated CryoStor™ with red blood, muscle, kidney and ovarian cells, amongst others. Results indicate CryoStor™ significantly improves post-thaw cell survival, provides more rapid recovery, higher yields and faster cell attachment and an enhanced cell survival at reduced cryoprotectant concentration [6,7], by maintaining the viability and health of the cells during freezing, transportation and storage, and thereby reducing cryopreservation-induced cell damage.

The aim of this study was to establish first whether CryoStor™ is effective in cryopreserving HSC from CB, and secondly whether its use would be beneficial in increasing the quality and viability of the final stem cell product available for transplantation and/or stem cell expansion.

Methods

All procedures and evaluations (apart from CB collection) were carried out by one of the authors to avoid technician related variability.

CB collection

CB used in this study was collected under a protocol approved by the Institutional Ethics Committee for the Sydney Cord Blood Bank (Sydney, Australia).

Processing

CB units were processed within 36 h of collection. CB was centrifuged and volume reduced (Optipress II, Baxter Healthcare, IL, USA) to obtain a buffy coat, using standard procedures established for CB banking [8]. Samples from the buffy coat were removed for NC and $CD34^+$ count and $CD34^+$ viability testing.

Cryopreservation

Equal volumes of DMSO (Wak-Chemie Medical GMBH, Steinbach, Germany) and dextran-40 (Baxter Healthcare, IL, USA) were mixed in a sterile 10-mL tube and allowed to cool for approximately 30 min in a refrigerator. CryoStor™ in 10% DMSO (CS10) was kept refrigerated until used. Buffy coat, 1.0 mL, from each individual CB unit was placed into each of three cryovials. Cryovial A (10% DMSO/dextran-40) had 0.25 mL DMSO/dextran-40 slowly added using a pipette. Cryovial B (10% DMSO/CryoStor™) had 1.0 mL CS10 slowly added, followed by 0.1 mL 100% DMSO, to give a final DMSO concentration of 9.5% in the total frozen product. Cryovial C (5% DMSO/CryoStor™) had 1.0 mL CS10 slowly added using a pipette.

Cryovials were mixed thoroughly by inverting several times and then placed inside a pre-chilled freezing container and transported to a $-0°C$ freezer and left for 3 h, using a validated freezing procedure [9] The cryovials were then transferred to vapor phase nitrogen at a temperature of $<-50°C$.

Thawing

After a minimum of 72 h, vials from each CB donation were simultaneously thawed in a 37°C water bath, placed on a rotor and mixed for 2 min.

Testing

Samples were removed and immediately analyzed for NC and $CD34^+$ count, $CD34^+$ viability and CFU assay. NC count was measured using a Sysmex K-4500 hematology analyze (Sysmex Corp, Kobe, Japan).

For the $CD34^+$ count, cells were incubated at a concentration of 5×10^5 with CD45–APC and CD34–PE Ab for 15 min, then 200 μL Annexin-V binding buffer was added along with 5 μL 7-AAD and 5 μL Annexin-V–FITC, (BD, Biosciences, CA, USA) and incubated for a further 15 min. Afterwards 500 μL ammonium chloride was added to lyse any remaining red cells. The labeled cells were run on a FacsCanto flow cytometer (BD, Biosciences, CA, USA). The results were analyzed using the ISHAGE gating strategy (10); the absolute $CD34^+$ count was obtained by multiplying the percentage of $CD34^+$ cells by the total nucleated cell count (dual platform).

CD34- viability was analyzed by flow cytometry within an hour of thawing, using the DNA-binding dye 7-AAD and Annexin-V. Percentage viability was taken as those cells that were negative for 7-AAD and Annexin-V.

Clonogenic assays were set up using Methocult H4434 (StemCell Technologies, Vancouver, Canada) and plated on 0.5-mL culture wells (Nunc, Roskilde, Denmark) at a concentration of 1.25×10^4 nucleated cells/well. A total of three wells per test sample was set up. The wells were left in a humidified 37°C incubator with 5% CO_2 for 14 days,

Table 1. Post-thaw results (mean ± 1 SD)

	10% DMSO/dextran-40	10% DMSO/CryoStor™	5% DMSO/CryoStor™
Nucleated cell recovery	91.2% ± 6.0	98.0% ± 4.9 $P < 0.001$	97.9% ± 6.1 $P < 0.001$
CD34$^+$ recovery	70.4% ± 15.5	87.2% ± 22.3 $P < 0.001$	75.2% ± 24.1 $P = 0.191$
Viable CD34$^+$ cells × 10^6	4.20 ± 2.90	5.2 ± 3.8 $P < 0.001$	4.5 ± 3.7 $P = 0.210$
CFU/1.25 × 10^4 cells plated	10.4 ± 10.0	16.7 ± 9.5 $P < 0.001$	17.4 ± 12.8 $P < 0.001$

at which time colonies containing greater than 40 cells were counted using an inverted microscope. The average of the three wells was taken and used for the analysis. The total number of colonies is reported in this study.

Statistical analysis

A two-tailed Student's *t*-test was used to analyze the results using the statistical program SPSS-12. (SPSS Inc., IL, USA).

Results

A total of 30 CB donations was processed for this study. Mean CB volume was 77 mL (range 49–129 mL), NC count was 19.4 × 10^6/mL (range 6.5–40.4 × 10^6/mL) and CD34$^+$ viability was 99.3% (range 96.1–100%). The overall results are summarized in Table 1.

NC recovery

Both 10% DMSO/CryoStor™ and 5% DMSO/CryoStor™ resulted in significantly superior NC recovery in comparison with 10% DMSO/dextran-40, with *P* values < 0.001 (Figure 1).

CD34$^+$ recovery

Higher CD34$^+$ recoveries were obtained from both 10% DMSO/CryoStor™ and 5% DMSO/CryoStor™; however, the latter was not statistically significant (Figure 2).

CD34$^+$ viability

Both 10% DMSO/CryoStor™ and 5% DMSO/CryoStor™ produced higher viable CD34$^+$ numbers; only 10% DMSO/CryoStor™ was statistically significant.

Clonogenic assay

A greater number of colonies was produced from cells cryopreserved with CryoStor™, in both 10% and 5% DMSO; these results were statistically significant, $P < 0.001$ (Figure 3).

Discussion

Although cryopreservation of HSC has been performed for more than 30 years, new approaches to cryobiology need to be explored to optimize cell retrieval.

In this study we investigated the effects of a new extracellular cryoprotectant (CryoStor™) in freezing CB HSC. While CryoStor™ has been tested with a range of non-hematopoietic cells and tissues, CryoStor™ has not previously been tested on HSC, either from CB or BM. This study shows that CryoStor™, rather than dextran-40, results in significantly higher NC, CD34$^+$, viable CD34$^+$

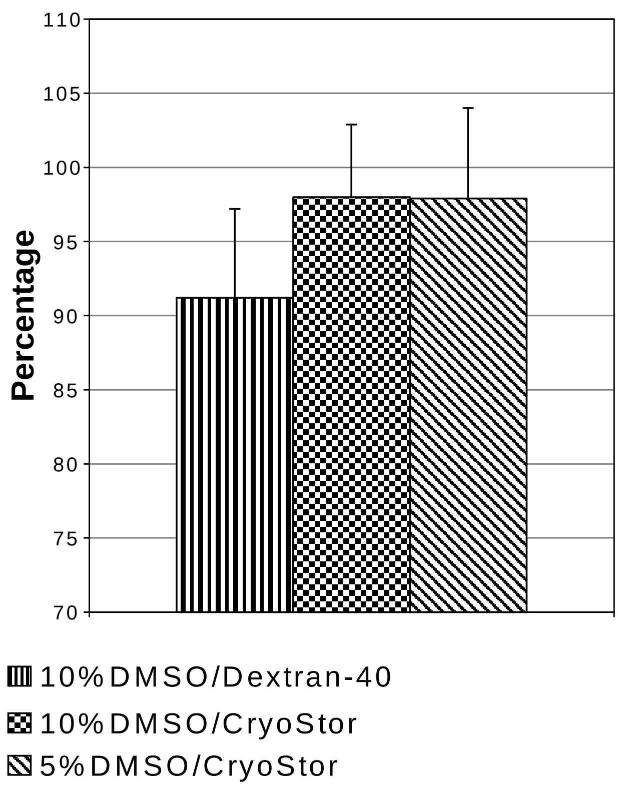

Figure 1. Post-thaw nucleated cell recovery.

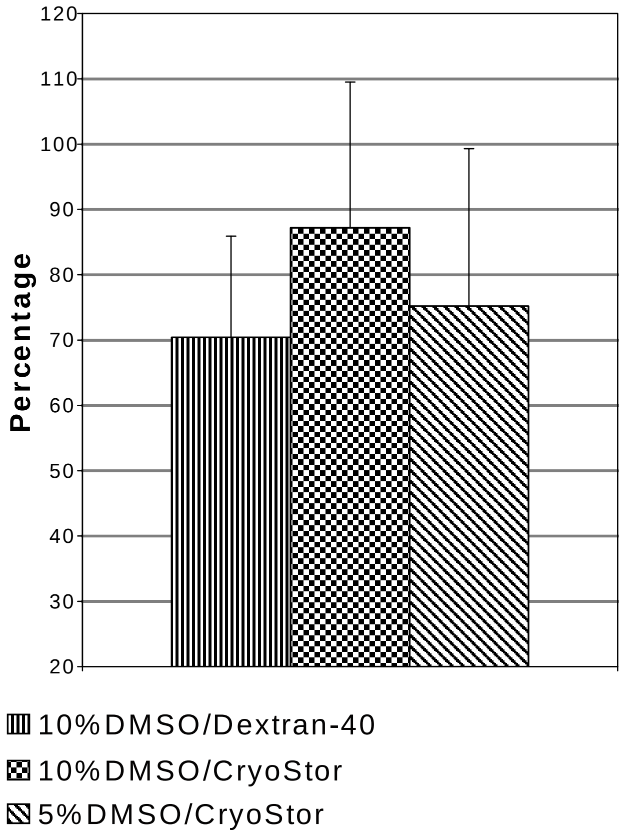

Figure 2. *Post-thaw CD34$^+$ recovery.*

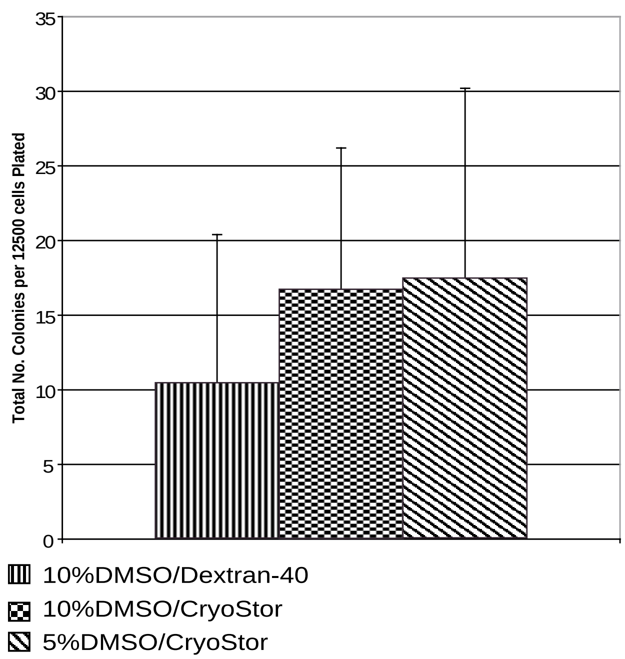

Figure 3. *Post-thaw total CFU.*

and CFU recovery when used with 10% DMSO, and a significantly higher NC and CFU recovery when used with 5% DMSO. This implies that there was less damage caused to cells when undergoing the freeze/thaw process, thus resulting in greater numbers of viable stem cells available for transplantation.

Clinical experience with CB transplantation has shown that cell number per kilogram body weight transplanted correlates with speed of hematopoietic reconstitution [1–3]. Loss of stem cell numbers and quality (function) after cryopreservation may impact on hematologic recovery after transplant. Thus, the presence of greater numbers of viable cells present in the product may improve hematopoietic reconstitution after transplant.

Reduction in the amount of DMSO used in cryopreservation of CB stem cells may reduce damage suffered by cells when not in the frozen state. This study supports the conclusions of others using DMSO, that a lower concentration of DMSO (5%) may be as effective as 10% in preserving cells during the freeze/thaw process [4]. The transfusion of less DMSO into patients may have the advantage of decreasing adverse reactions. Additionally, reducing damage to stem cells will result in a higher number of viable cells available for *ex vivo* expansion of CB programs. Better recovery of stem cells after thawing may also be an important issue in the future development of *ex vivo* stem cell expansion research.

Disclaimer

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