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Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood ☆

J.P. Rodrigues [a,b], F.H. Paraguassú-Braga [b,c], L. Carvalho [a], E. Abdelhay [c], L.F. Bouzas [b,c], L.C. Porto [a,*]

[a] *Histocompatibility and Cryopreservation Laboratory, Department of Histology and Embryology, Institute of Biology Roberto Alcantara Gomes, Rio de Janeiro State University, Av Marechal Rondon 381 Terreo, São Francisco Xavier, 20950-003 Rio de Janeiro, Brazil*
[b] *Umbilical Cord Blood Bank, Bone Marrow Transplantation Unit, National Cancer Institute, Rio de Janeiro, Brazil*
[c] *Bone Marrow Transplantation Unit, National Cancer Institute, Rio de Janeiro, Brazil*

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Abstract

Bone marrow transplantation (BMT) is a therapeutic procedure that involves transplantation of hematopoietic stem cells (HSC). To date, there are three sources of HSC for clinical use: bone marrow; mobilized peripheral blood; and umbilical cord blood (UCB). Depending on the stem cell source or type of transplantation, these cells are cryopreserved. The most widely used cryoprotectant is dimethylsulfoxide (Me_2SO) 10% (v/v), but infusion of Me_2SO-cryopreserved cells is frequently associated with serious side effects in patients. In this study, we assessed the use of trehalose and sucrose for cryopreservation of UCB cells in combination with reduced amounts of Me_2SO. The post-thawed cells were counted and tested for viability with Trypan blue, the proportion of HSC was determined by flow cytometry, and the proportion of hematopoeitic progenitor cells was measured by a colony-forming unit (CFU) assay. A solution of 30 mmol/L trehalose with 2.5% Me_2SO (v/v) or 60 mmol/L sucrose with 5% Me_2SO (v/v) produced results similar to those for 10% (v/v) Me_2SO in terms of the clonogenic potential of progenitor cells, cell viability, and numbers of $CD45^+/34^+$ cells in post-thawed cord blood cryopreserved for a minimum of 2 weeks. Thus, cord blood, as other HSC, can be cryopreserved with 1/4 the standard Me_2SO concentration with the addition of disaccharides. The use of Me_2SO at low concentrations in the cryopreservation solution may improve the safety of hematopoietic cell transplantation by reducing the side effects on the patient.

Keywords: Cryopreservation; Dimethylsulfoxide; Hematopoietic stem cells; Sucrose; Trehalose

Hematopoietic stem cell transplantation (HSCT) is a medical therapeutic procedure which aims to reconstitute the hematopoietic activity of bone marrow. It is the first treatment option for many bone marrow diseases such as bone marrow aplasia or leukemia, and an adjuvant tool in the treatment of solid tumors. As an adjunct in the treatment of malignancies, HSCT offers hope in cancer treatment as new and more aggressive therapies are used [6]. Stem cells from bone marrow (BM), mobilized peripheral blood (MPB) or umbilical cord blood and from different donor types (autologous, syngeneic or allogeneic) are used for these transplants [32]. Often these transplant modalities require cryopreservation and storage of hematopoietic stem cells (HSC). This is especially true of umbilical cord blood stem cells, which need to remain cryopreserved in umbilical cord blood banks for possible future use.

Umbilical cord blood (UCB) is a valuable source of stem cells in the treatment of hematologic, oncologic, immunologic and metabolic diseases [4,12,20]. Most UCB units are stored in liquid nitrogen in individual bags for several years and quickly thawed just prior to transplantation. A single unit contains a limited number of hematopoietic progenitors, although with high proliferation and repopulating

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* Corresponding author. Fax: +55 21 2587 8164.
E-mail address: lcporto@uerj.br (L.C. Porto).

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capabilities [33,34]. UCB transplant provides the advantages of requiring less stringent HLA matchings between donors and recipients as well as a lower severity of graft versus host disease (GVHD) after transplant. However, it is associated with higher morbidity and mortality due to delayed neutrophil (median >25 days) and platelet (median >80 days) engraftments, essentially as a result of the limited dose of HSCs in UCB when compared to transplants using BM or MPB as HSC sources [20,19,29].

Optimization of cryopreservation protocols to maintain the quality of HSCs has been an important task for UCB banks. To allow this prolonged storage, the HSC product is slowly cooled at a controlled rate and stored at −196 °C in liquid nitrogen. Cooling the cells slowly avoids intracellular ice buildup, which can cause rupture of the cell membrane. Nevertheless, it can result in dehydration of the cells by formation of extracellular ice. To prevent this, a cryoprotectant is added. The most widely used cryoprotectant is dimethylsulfoxide (Me_2SO) that is a hygroscopic polar compound developed originally as a solvent for chemicals. Its properties were described originally in 1959 by Lovelock et al. [16].

Cryopreserved HSC products can be stored for years [30]. The presence of cryoprotectant and changes resulting from the freezing and thawing process require special precautions during and after the infusion of HSC product into the patient [25].

Several side effects have been described during the infusion of Me_2SO into patients, including sedation, headache, nausea, vomiting, hypertension, bradycardia, hypotension or anaphylactic shock [26]; effects on the blood include intravascular hemolysis, hyperosmolality, and increased serum transaminase levels after IV administration in humans [22,23,35].

Disaccharides such as sucrose and trehalose have been widely used as natural cryoprotectants [17]. In nature, many organisms can survive in an inert, desiccated state for extended periods with up to 99% water loss, in a phenomena known as anhydrobiosis [9,18], which is much similar to the phenomena that occurs during cryopreservation and related in some instances to the accumulation and storage of saccharides, such as trehalose. Trehalose may also be used in parenteral nutrition. Moreover, the combination of catalase and trehalose as a supplement in conventional freezing medium resulted in better protection of growth factor receptors, adhesion molecules, and functionality of hematopoietic cells [24,36].

In this study, we assessed the ability of two disaccharides, trehalose and sucrose, to enhance the cryopreservation of UCB. Both sugars enabled the concentration of Me_2SO to be lowered in the infusion product of the HSCT.

Materials and methods

Umbilical cord blood

Umbilical cord blood units were obtained according to the local procedures of the UCBB of the National Cancer Institute (INCA-Brazil). All procedures were approved by the institutional ethics committee.

UCB collection and processing

Pregnant women were recruited by interview to donate their child's cord blood before delivery. Potential donors were screened with a clinical questionnaire in the maternity hospital. UCB units were collected from eligible mothers with an *ex-utero* procedure by the nurses from the UCBB working in the maternity hospital for that purpose. Briefly, after natural delivery of the placenta, the umbilical cord and placenta were sent to the collection room where the placenta was hung up, allowing the umbilical cord to be cleaned. After this procedure, the cord blood was collected by gravity into blood collection bags, containing CPDA-1 anticoagulant solution, by insertion of a needle into the umbilical vein. After collection, the bag was sealed and sent to the processing facility of the INCA Cord Bank. Before processing, cells were counted in a sample of UCB using a Cell-Dyn 1700 (Abbott Ltda, Sao Paulo, SP, Brazil) and then the sample was mixed with hydroethylstarch in a 4:1 ratio and centrifuged at 59*g* for 8 min. The white blood cell (WBC)-rich plasma was collected in a separate bag and centrifuged at 500*g* for 12 min [14]. UCB was processed up to 24 h from delivery of the placenta.

Cell lines

The cell lines KG1, K562 and S17 were obtained from the Rio de Janeiro Cell Bank (APABCAM, Rio de Janeiro, RJ, Brazil) and maintained in our lab according to standard protocols, in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and L-glutamine (Sigma–Aldrich Corp., St. Louis, MO, USA) 1 mmol/L.

Cryopreservation and thawing

In a pilot study, the best concentrations of Me_2SO, sucrose, trehalose, and their combinations were determined using hematopoietic cells lines KG1, K562 and bone marrow stromal cell line S17. Forty-three different combinations of cryoprotectants were tested with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay, and 10 combinations were chosen for use with UCB cells. A Me_2SO 10% (v/v) solution was used as the standard cryopreservation solution (100% viability) and an aliquot of cord blood cells without any cryoprotectant was used as a negative control (0% viability).

Preparation of cryoprotectant solutions

All solutions were prepared in previously marked cryovials just prior to use and before addition of cells. After addition of the cells, all the vials had 1 mL of final suspension. Dextran 40 (Fresenius KABI, Rio de Janeiro, RJ,

Brazil) (10% v/v solution in NaCl 0.9%) was used to dilute the Me_2SO and 1% human serum albumin (HSA) (Invitrogen, Carlsbad, CA, USA).

Different amounts of disaccharides and Me_2SO were added to the cryovials in accordance with the requested concentration. The solutions were then stored in the refrigerator until the cells were added.

WBC-rich plasma from 5 placentas was pooled and two cell counts were performed. 5×10^6 cells in 100 μL were transferred to a 1-mL cryovial containing each cryoprotectant solution, and the cryovial was immediately frozen in a pre-established program in a Controlled Rate Freezing System (Custom Biogenic Systems, Shelby Township, MI, USA): Ramp 1, −5 °C per minute to 4 °C; Ramp 2, equilibrate at 4 °C for 10 min; Ramp 3, −2 °C per minute to −40 °C; Ramp 4, −10 °C per minute to −80 °C; and Ramp 5, equilibrate at −80 °C for 10 min. After the end of the freezing cycle, the samples were stored under liquid nitrogen for a minimum of 2 weeks before thawing and further analysis.

Samples were thawed by rapidly immersing the cryovials in a 37 °C water bath, and then divided for the different analyses: samples were counted and tested for cell viability with Trypan blue; the number of $CD45^+/CD34^+$ cells was measured by flow cytometry analysis, and the hematopoietic progenitor content was accessed by a colony-forming units (CFU) assay.

Trypan blue viability assay

Viability was accessed with a Trypan blue (Sigma–Aldrich Corp., St. Louis, MO, USA) dye exclusion assay. A 1:1 dilution of the suspension was prepared using a 0.4% Trypan blue solution in PBS and then 1 mL of a red cell lysis solution (0.5 M acetic acid) was added. Viability was expressed as the percentage of viable unstained cells in suspension related to the total number of cells counted in a hemocytometer counting chamber. Counts were done in triplicate.

Flow cytometry analysis

$CD45^+/CD34^+$ cell enumeration was done with a single platform protocol and gating strategy based on International Society for Cellular Therapy (formerly ISHAGE-ISCT) guidelines following the method described by Keeney et al [13]. Briefly, 2×10^6 cells/mL were incubated with phycoerythrin-conjugated anti-CD34 (8G12 clone) and fluorescein isothiocyanate-conjugated anti-CD45 (2D1 clone) monoclonal antibodies (BD Biosciences, San Jose, CA, USA). The percentage of $CD34^+$ cells was determined in a FACSCAN (BD Biosciences, San Jose, CA, USA) with initial gating on $CD45^+$ cells. To avoid damage to cells related to Me_2SO treatment, all post-thawed samples were washed with 10 mL of 2.5% human albumin and dextran 40 (10% in NaCl 0.9%) in a 1:1 ratio solution before the staining. Tests were performed in triplicate.

Colony-forming unit (CFU) assays

CFU assays were carried out as described by Limaye et al. [15]. Briefly, 5×10^4 cells/ml were plated (in duplicate) in methylcellulose medium containing a combination of growth factors (Methocult, Stem Cell Technologies Inc, Vancouver, BC, Canada), i.e. recombinant human (rHu), GM-CSF (2 ng/mL), rHuIL3 (4 ng/mL), rHuSCF (20 ng/mL), and rHuEPO (2 U/mL). The plates were incubated along with sterile water-containing plates for 14–16 days in 5% laboratory CO_2 in 300/3000 Series Incubators (Revco, Ashille, Ohio, USA). After this time, the colonies were scored under an inverted microscope (Leica DMIL, Leica Microsystems, AG, Wetzlar, Germany) for burst-forming units-erythroid (BFU-E), colony-forming units-erythroid (CFU-E) and colony-forming units-granulocyte-macrophage (CFU-GM).

Statistical analysis

All data were analyzed with a statistical software package (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Kruskal–Wallis one-way ANOVA test was used for comparisons of groups of more than two samples. p values less than 0.05 was considered significant.

Results

Screening with cells line

Of 43 different solutions tested in a pilot study for cryopreservation of three cells lines, eight were chosen based on their performance for further testing with cord blood samples. These were: 10%, 5%, and 2.5% (v/v) Me_2SO; 2.5% (v/v) Me_2SO plus 30 mmol/L trehalose; 2.5% (v/v) Me_2SO plus 30 mmol/L of sucrose; 5% (v/v) Me_2SO plus 15 mmol/L of trehalose; 5% (v/v) Me_2SO plus 15 mmol/L of sucrose; 5% (v/v) Me_2SO plus 60 mmol/L of trehalose; 5% (v/v) Me_2SO plus 60 mmol/L of sucrose; and solution without cryoprotectant (Table 1).

Trypan blue viability assay

No significant differences in the percentage viability were detected among the cryopreservation solutions with 2.5%, 5% and 10% (v/v) Me_2SO independent of the addition of trehalose or sucrose. When cells were cryopreserved without any cryoprotectant, fewer than 60% of the UCB nucleated cells were viable (Fig. 1).

Flow cytometry with $CD45^+/CD34^+$

The percentages of $CD34^+$ cells retrieved in the post-thawed UCB cryopreserved with different concentrations of Me_2SO [10%, 5% and 2.5% (v/v)] without disaccharides were very similar. Addition of 30 mmol/L trehalose or sucrose to a solution with 2.5% (v/v) Me_2SO resulted in a

Table 1
Solutions and cell lines in the pilot study

Solution	Me_2SO (%v/v)	Sucrose (mmol/L)	Trehalose (mmol/L)	% Viability		
				K562	KG1	S17
1	0	0	0	12.5 ± 0.8	13.6 ± 1.2	11.5 ± 0.6
2	1	0	0	33.4 ± 2.5	35.8 ± 2.6	36.4 ± 0.3
3	2.5	0	0	65.0 ± 2.8	64.2 ± 3.3	66.2 ± 0.7
4	5	0	0	82.1 ± 0.7	80.5 ± 0.9	86.4 ± 0.0
5	10	0	0	100.0 ± 0.9	100.0 ± 1.4	100 ± 2.3
6	0	15	0	36.5 ± 0.8	35.2 ± 0.7	35.5 ± 0.3
7	0	30	0	30.0 ± 1.5	29.6 ± 3.5	31.5 ± 0.1
8	0	45	0	17.0 ± 0.7	16.2 ± 1.7	18.6 ± 0.2
9	0	60	0	19.3 ± 0.9	18.2 ± 5.7	18.9 ± 0.7
10	0	90	0	16.3 ± 0.4	14.9 ± 2.7	20.0 ± 0.3
11	0	120	0	38.1 ± 1.5	37.8 ± 0.6	46.2 ± 0.8
12	0	180	0	25.8 ± 2.6	26.4 ± 9.1	24.3 ± 1.0
13	0	0	15	37.1 ± 1.6	36.1 ± 2.3	38.2 ± 0.7
14	0	0	30	23.9 ± 1.3	22.7 ± 1.1	21.3 ± 3.0
15	0	0	45	16.9 ± 1.2	15.7 ± 1.6	19.4 ± 1.6
16	0	0	60	15.2 ± 2.2	16.9 ± 6.7	10.8 ± 2.2
17	0	0	90	18.5 ± 4.0	17.6 ± 3.2	19.4 ± 0.9
18	0	0	120	31.6 ± 3.8	34.0 ± 3.3	33.1 ± 0.5
19	0	0	180	26.4 ± 1.7	27.1 ± 5.8	26.4 ± 0.5
20	1	15	0	33.5 ± 2.4	32.5 ± 2.4	33.9 ± 1.8
21	1	30	0	24.7 ± 1.2	22.6 ± 6.8	28.3 ± 2.3
22	1	60	0	42.3 ± 0.5	41.8 ± 1.5	45.1 ± 4.6
23	1	0	15	41.2 ± 1.4	40.3 ± 2.8	42.5 ± 0.7
24	1	0	30	29.9 ± 1.6	28.3 ± 1.2	27.6 ± 0.0
25	1	0	60	37.1 ± 2.8	36.4 ± 5.5	39.0 ± 0.5
26	2.5	15	0	66.3 ± 3.4	65.6 ± 3.9	67.1 ± 0.9
27	2.5	30	0	96.3 ± 1.1	92.3 ± 3.3	89.8 ± 4.8
28	2.5	60	0	68.8 ± 0.9	67.4 ± 1.0	65.9 ± 3.3
29	2.5	0	15	64.0 ± 1.8	63.2 ± 2.4	63.8 ± 1.2
30	2.5	0	30	86.1 ± 0.8	85.4 ± 4.2	86.0 ± 1.3
31	2.5	0	60	68.5 ± 0.6	65.2 ± 3.4	63.2 ± 0.6
32	5	15	0	90.0 ± 0.4	91.8 ± 2.6	92.5 ± 0.2
33	5	30	0	75.4 ± 0.5	66.5 ± 0.9	74.1 ± 5.7
34	5	60	0	92.5 ± 1.3	91.5 ± 0.8	90.9 ± 0.5
35	5	0	15	83.2 ± 0.6	89.0 ± 1.5	85.2 ± 1.0
36	5	0	30	73.2 ± 3.4	70.2 ± 0.7	71.5 ± 0.5
37	5	0	60	89.6 ± 1.1	90.3 ± 0.9	93.1 ± 3.3
38	10	15	0	90.1 ± 1.1	92.7 ± 0.4	93.7 ± 3.5
39	10	30	0	96.4 ± 1.3	94.3 ± 1.6	98.2 ± 8.0
40	10	60	0	98.6 ± 0.4	97.5 ± 1.3	96.4 ± 1.1
41	10	0	15	92.5 ± 0.7	93.4 ± 1.2	93.7 ± 6.2
42	10	0	30	98.5 ± 1.8	90.7 ± 2.2	94.5 ± 7.2
43	10	0	60	94.3 ± 0.5	89.9 ± 6.0	86.4 ± 2.9

Values are expressed as percentage of the viability afforded by the control cryoprotectant solution, 10% Me_2SO (v/v). Solutions 1, 3, 4, 5, 27, 30, 32, 34, 35 and 37 were chosen based on their performance with these cells lines for further testing with cord blood samples.

higher percentage of $CD34^+$ cells. Variable results were observed with cryopreservation solutions containing 5% (v/v) Me_2SO when trehalose or sucrose was added at 15 mmol/L or 60 mmol/L, but no significant differences were detected in the levels of $CD34^+$ cells among these cryopreservation solutions. The percentage of $CD34^+$ was significantly reduced when the cells were cryopreserved without Me_2SO ($p < 0.01$) (Fig. 2).

Colony-forming unit (CFU) assays

The influence of the cryopreservation solutions on the clonogenic capacities of post-thawed UCB evaluated as the mean number of CFU is shown in Table 2. UCB cells cryopreserved without Me_2SO were not capable of forming colonies.

Samples cryopreserved with 2.5% (v/v) Me_2SO without disaccharides showed a significant reduction in the total number of CFU (42%) when compared with 10% (v/v) Me_2SO, our control ($p < 0.01$). Addition of 30 mmol/L trehalose to the 2.5% (v/v) Me_2SO solution restored the total number of CFU to control levels; this restorative effect was also seen in the case of CFU-GM with >1000 cells. Addition of 15 mmol/L trehalose to the 5% (v/v) Me_2SO solution increased the number of CFU-GM with >1000 cells by 46% and 19% in comparison with 10% (*v*/*v*) Me_2SO

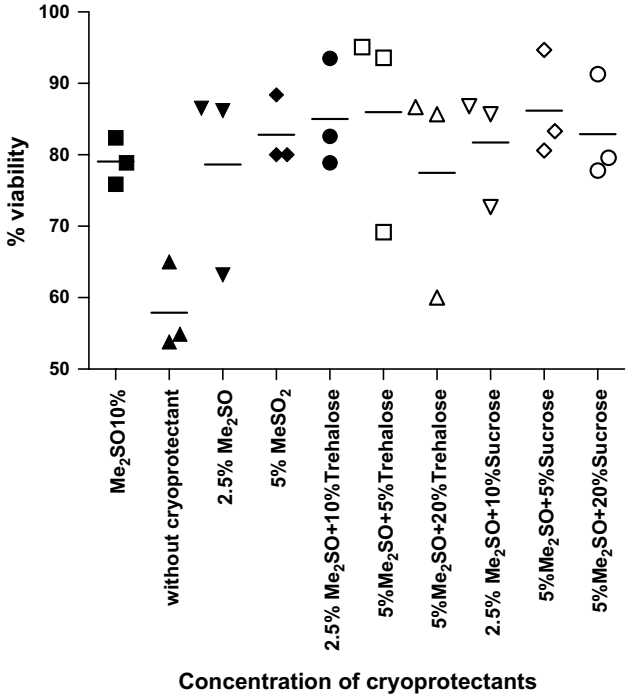

Fig. 1. Percentage of UCB cell viability quantified by Trypan blue dye exclusion assay. Viable cells exclude the dye and therefore remain colorless while nonviable cells take up the dye and stain blue. The graph shows the viability of UCB cells after cryopreservation and thawing with different compositions and concentrations of cryoprotectants.

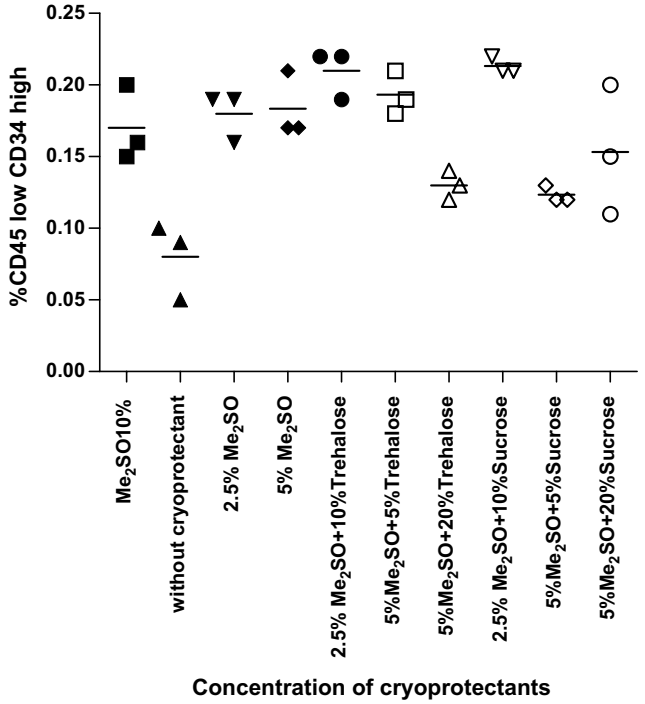

Fig. 2. Flow cytometric analysis of post-thawed UCB cells. The detection of the cells expressing both the $CD34^+$ antigen and the $CD45^+$ pan-leukocyte antigen can be used to measure the subpopulations of hematopoietic stem and progenitor cells. A single-platform flow cytometric method is available for rapid determination of absolute $CD34^+$ cells, based on guidelines developed for the International Society of Hematotherapy and Graft Engineering (ISHAGE). The graph shows the absolute number $CD34^+$ cells cryopreserved with different compositions and concentrations of cryoprotectants.

and 5% (*v*/*v*) Me_2SO, respectively. The observed increase in clonogenic capacity of the cryopreserved cells in relation to 10% (v/v) Me_2SO was statistically significant ($p < 0.05$). The solutions with 5% (v/v) Me_2SO had similar results towards our control, without any statistically significant difference.

The results for sucrose were not as encouraging. We expected to see a gradual increase in the number of CFU in the presence of 5% (*v*/*v*) Me_2SO and increasing concentrations of sucrose. However, the addition of sucrose to 15 mmol/L resulted in a lower recovery of CFU,compared to 5% (v/v) Me_2SO alone, and similar to that for 2.5% (v/v) Me_2SO alone (Table 2). This difference appears to be related to the reduced survival of the erythroid colonies (BFU-E and CFU-E). Only at 60 mmol/L in 5% (v/v) Me_2SO did sucrose yield a similar number of colonies as 10% (v/v) Me_2SO alone.

The clonogenic potential of UCB cells to form GM colonies with >1000 cells was improved by addition of 30 mmol/L trehalose or 30 mmol/L sucrose to the 2.5% (v/v) Me_2SO solution, resulting in colony numbers not significantly different from those for 10% (v/v) Me_2SO. Curiously, 60 mmol/L sucrose had a beneficial effect on the number of GM colonies with <1000 cells compared to 10% (v/v) Me_2SO, albeit not significant; however, when compared with the 5% (v/v) Me_2SO solution, this difference is significant, with a 77% increase ($p < 0.01$).

Discussion

HSC transplantation is an established therapeutic approach for a number of hematological disorders, both malignant and non-malignant [32]. In the setting of HSC transplantation, the interval between collection of HSCs and transplantation varies from several days to weeks (BM, PBPC) or even years with UCB [11]. Here we analyzed the capacity of two disaccharides, trehalose and sucrose, to maintain the quality of HSCs from UCB with the aim of eliminating or reducing the amount of Me_2SO in the infusion product and thus the need for risky Me_2SO wash procedures. Complications related to the presence Me_2SO during re-infusion of cryopreserved hematopoietic stem cells have been described [25,1].

Disaccharides such as sucrose, lactose, and trehalose have been widely used as cryoprotectants, as excipients for freeze drying, and as stabilizers during dehydration. For example, liposomes dried and rehydrated without the addition of disaccharides suffer damage and leak their contents to the surrounding medium, while those dried with a disaccharide retain their contents. Plant seeds, yeast, and brine shrimp accumulate large amounts of disaccharides, especially sucrose and trehalose [5]. The stabilizing effect of trehalose has led to its recent use in a number of

Table 2
Clonogenic capacities of post-thawed CB HSC

	CFU	BFU-E	CFU-E	CFU-GM > 1000 cells	CFU-GM < 1000 cells
10.0% Me_2SO (Control)	52.5 ± 2.4	17.2 ± 0.7	17.2 ± 4.6	6.5 ± 1.0	11.5 ± 3.3
5.0% Me_2SO	50.2 ± 2.5	17.2 ± 3.8	15.7 ± 3.1	8.0 ± 1.8	9.3 ± 1.5
2.5% Me_2SO	30.0 ± 1.2*	8.5 ± 0.7	12.0 ± 2.1	3.0 ± 0.0*	6.5 ± 0.9
2.5%Me_2SO + 30 mM trehalose	51.0 ± 0.5**	19.7 ± 0.3	19.7 ± 1.0	4.7 ± 0.3**	6.8 ± 0.3
5.0% Me_2SO + 15 mM trehalose	56.0 ± 0.5***	14.2 ± 0.3	22.5 ± 0.7	9.5 ± 0.9***	9.8 ± 0.3
5.0% Me_2SO + 60 mM trehalose	54.0 ± 2.1	15.7 ± 0.3	19.5 ± 0.7	6.1 ± 1.3	12.5 ± 2.2
2.5% Me_2SO + 30 mM sucrose	40.0 ± 2.2**	12.7 ± 4.6	12.0 ± 2.8	6.5 ± 0.5**	9.2 ± 0.8
5.0% Me_2SO + 15 mM sucrose	33.0 ± 1.0	6.0 ± 0.0	12.0 ± 0.0	5.5 ± 0.5	9.5 ± 1.5
5.0% Me_2SO + 60 mM sucrose	54.6 ± 2.8	13.7 ± 1.0	17.7 ± 0.3	6.7 ± 2.1	16.5 ± 1.7

Values are expressed as mean number ± SD of colony forming units, Me_2SO (v/v).
* Significantly different from 10% Me_2SO ($p < 0.05$).
** With the addition of the disaccharide, the result was similar to that of the control ($p > 0.05$). Without disaccharide, these solutions gave unsatisfactory results.
*** With the addition of the disaccharide, the result was better than that of the control ($p < 0.05$).

biomedical, cosmetics, and pharmaceutical applications, including as an ingredient of nontoxic cryoprotective solutions for vaccines, many types of cells, and organs for surgical transplant [7].

In the present study, analysis of the total number of CFU and CFU-GM with >1000 cells from UCB shows that 30 mmol/L of disaccharides added to a 2.5% (v/v) Me_2SO solution gives a recovery of clonogenic potential similar to that produced by 10% (v/v) Me_2SO, demonstrating the potential use of these sugars. The primary objective of this work was to evaluate the cryoprotectant effect of trehalose in the HSCs of UCB; however, the results also suggest that 60 mmol/L sucrose added to 5% (v/v) Me_2SO gives clonogenic characteristics similar to that of 10% (v/v) Me_2SO. Moreover, a curious 28.7% increase in the number of GM colonies was observed mainly for GM colonies with fewer than 1000 cells.

The precise mechanism by which disaccharides act to preserve biological systems during freezing and drying is not well understood [31]. It is currently believed that effective cryoprotectants for proteins or enzymes in solution are excluded from the immediate vicinity of these biological molecules. This argument has also been extended to membranes [10]. This mode of action should be contrasted with that proposed to explain the effectiveness of disaccharides for lyophilization of liposomes and cellular organisms; for such systems it has been argued that, in the absence of water, disaccharides lower the melting temperature of bilayer membranes, thereby preventing leakage during freezing, drying, and rehydration [10]. There are many protection mechanisms proposed for the action of trehalose: it acts as a compatible solute, it is an excellent protector of membranes and proteins, and it protects the membranes during dehydration by hydrogen bonding to the phospholipid head groups [8]. This interaction increases head-group spacing, hence lowering the transition temperature of the phospholipids [21]. Different ways are proposed to explain how trehalose might protect proteins. Timasheff and his colleagues provided evidence that the disaccharides stabilize proteins during cooling, because the disaccharides are preferentially excluded from the surface of the proteins in aqueous solution [2].

In this work, solutions containing disaccharides and lower concentrations of Me_2SO showed better colony-forming potential than the control solution, with similar number of viable cells. Water-soluble, cryoprotective macromolecules such as albumin, dextrans, modified gelatins, polyvinylpyrrolidone, polyethylene oxide, polyethylene glycol, and hydroxyethyl starch (HES) exhibit the principal advantage of not entering into the cells [27]. This property significantly facilitates removal of the cryoprotectant after thawing. HES-cryopreserved red blood cells (RBC) in patients have been assayed, and although hemoglobin increased after transfusion, the levels normalized within 24 h. The HES was eliminated from the plasma following first-order kinetics [28]. The data indicate that the transfusion of one autologous unit of RBC after cryopreservation with HES is safe and well tolerated. Further investigations are necessary to evaluate the effects of biodegradable large compounds and their tolerance by patients treated with UCB units cryopreserved with such large molecular-weight compounds. Unlike Me_2SO, which penetrates into the cell membrane to produce its cryoprotective effect both extra- and intracellularly, the disaccharides do not penetrate the cell membrane, but act only at the extracellular surface of the cells. At this site, the disaccharides increase the osmolarity of the medium, keeping fewer water molecules from contacting the cell exterior. This reduces the formation of ice crystals in the interior of the cell during the freezing process, thus stabilizing the cellular membrane and proteins [3]. It can be suggested that if these disaccharides were also able to get into the cell interior, then they might produce even better cryoprotective results. Nevertheless, it was possible to reduce the Me_2SO concentration to 2.5% (v/v) by using either one of the disaccharides. Moreover, this concentration of Me_2SO should produce fewer side effects in patients. Trehalose has also been used to preserve RBC, and its use during cryopreservation reduced red cell lysis and the release of free hemoglobin into the medium, thus contributing to fewer side effects [5].

In conclusion, the results demonstrate that the disaccharides trehalose and sucrose can be used in cryopreservation solutions to reduce the concentration of Me_2SO from the current standard 10% (v/v) to 2.5% (v/v) with trehalose and 5% (v/v) with sucrose. Further studies shall address the long-term storage capabilities of disaccharides as cryoprotectants as well as their capabilities for preserving HSC for *in vivo* reconstitution in animal models.

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