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Evaluations of bioantioxidants in cryopreservation of umbilical cord blood using natural cryoprotectants and low concentrations of dimethylsulfoxide ☆

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ABSTRACT

Transplantation using hematopoietic stem cells from umbilical cord blood (UCB) is a life-saving treatment option for patients with select oncologic diseases, immunologic diseases, bone marrow failure, and others. Often this transplant modality requires cryopreservation and storage of hematopoietic stem cells (HSC), which need to remain cryopreserved in UCB banks for possible future use. The most widely used cryoprotectant is dimethylsulfoxide (Me_2SO), but at 37 °C, it is toxic to cells and for patients, infusion of cryopreserved HSC with Me_2SO has been associated with side effects. Freezing of cells leads to chemical change of cellular components, which results in physical disruption. Reactive oxygen species (ROS) generation also has been implicated as cause of damage to cells during freezing. We assessed the ability of two bioantioxidants and two disaccharides, to enhance the cryopreservation of UCB. UCB was processed and subjected to cryopreservation in solutions containing different concentrations of Me_2SO, bioantioxidants and disaccharides. Samples were thawed, and then analysed by: flow cytometry analysis, CFU assay and MTT viability assay. In this study, our analyses showed that antioxidants, principally catalase, performed greater preservation of: CD34+ cells, CD123+ cells, colony-forming units and cell viability, all post-thawed, compared with the standard solution of cryopreservation. Our present studies show that the addition of catalase improved the cryopreservation outcome. Catalase may act on reducing levels of ROS, further indicating that accumulation of free radicals indeed leads to death in cryopreserved hematopoietic cells.

Introduction

Transplantation using hematopoietic stem cells from umbilical cord blood (UCB) is a life-saving treatment option for patients with select oncologic diseases, immunologic diseases, hemoglobinopathies, and bone marrow failure syndromes [18]. The first UCB transplantation was performed in 1988 when a 6-year-old with Fanconi Anemia from the United States underwent a successful transplantation performed by Dr. Eliane Gluckman at the L'hospital St Louis in Paris. The source was HLA-identical UCB procured from his unaffected sibling [15].

In 1991, the New York Center established the first unrelated donor cord blood bank in the world [37]. In 1993, the first unrelated UCB transplantation in the world was performed on a young child with recurrent T-cell acute lymphoblastic leukemia utilizing a banked cord blood unit from the New York Blood center. In 1996, outcomes were reported for the 25 consecutive transplants with unrelated UCB at New York Blood center and transplanted at Duke University [24]. Important lessons learned from these early UCB transplantations are that utilization of HLA-mismatched UCB is acceptable (at least when matched at four of six HLA-A, -B, and –DRB1 antigens), and that the incidence of graft-versus-host disease (GvHD) is less than expected based on the degree of HLA mismatch [35,43]. Since the first human cord blood transplant was performed 20 years ago, [15] cord blood banks (CBB) have been established for related or unrelated UCB transplantation. CBBs around the world guard more than 400,000 available units and due to this, more than 20,000 successful umbilical cord blood transplants have been performed in children and in adults [16].

Often this transplant modality requires cryopreservation and storage of hematopoietic stem cells (HSC), which remain cryopreserved in UCB banks for their possible future use [36].

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

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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 agent 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 and Bishop [28].

The infusion of cryopreserved hematopoietic stem cell products with Me_2SO has been associated with side effects of nausea, chills, hypotension, dyspnea and cardic arrhythmia [2,12,44]. Studies of hematopoietic stem cell transplants in children show that reactions in this patient group are more severe [14,31], because they have low body weight and Me_2SO is dose dependent.

Disaccharides, such as sucrose and trehalose have been widely used as natural cryoprotectants, as expecipients for freeze drying, and stabilizers during dehydratation [32]. However, only few studies used disaccharides for cell therapy in transplantation of hematopoietic stem cell [36,42].

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 [8,33], which is much similar to the phenomena that occurs during cryopreservation and related in some instances to the accumulation and storage of disaccharides, such as trehalose.

There has been tremendous interest in the use of trehalose for stem cell preservation. Limaye and colleagues showed that catalase and trehalose were effective in preserving hematopoietic progenitor cells [27,38–40]. Scheinkonig and colleagues [42] also evaluated trehalose with (and without) insulin (to promote uptake of trehalose). They observed that 0.5 M trehalose added to a 10% Me_2SO solution was most effective at preserving colony-forming capability of bone marrow and peripheral blood stem cells, but there was no difference between the solutions containing insulin, and for those without.

With cryopreservation, cell membranes are maximally affected due to intracellular ice formation that takes place during freezing [3]. Generation of oxygen-free radicals is one of the several factors which damage cells during low-temperature storage [21,22]. Limaye and colleagues in 1997, showed that addition of certain bioantioxidants to the conventional freezing medium improves post-thaw cellular recovery [26].

Because cryopreservation is indispensable in the banking of UCB cells for their use in unrelated HLA matched procedure, optimization of freezing protocols to get improved recovery has been our goal. The use of membrane stabilizers and bioantioxidants has been shown to improve cryoprotection [27].

Hence, in this study, we chose to assess the ability of two bioantioxidants (catalase and ascorbic acid) and two disaccharides (trehalose and sucrose), to enhance the cryopreservation of UCB. Both, bioantioxidants and disaccharides enabled the reducing of the concentration of Me_2SO in the infusion product of the UBC [36,38].

Materials and methods

Umbilical cord blood

Two units of umbilical cord blood were obtained according to the local procedures of the UCB bank of the National Cancer Institute (INCA-Brazil). The institutional ethics committee approved all procedures.

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 UCB bank 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. Needles, connected to blood collection bags containing CPDA-1 anticoagulant solution, were inserted into the umbilical vein and left to allow the cord blood to collect by gravity. After collection, the bags were sealed and sent to the processing facility of the INCA Cord Bank. Before processing, cells were counted in a sample of UCB using an ABX Micros 60 (Horiba Ltda, Sao Paulo, SP, Brazil) and then the sample was mixed with hydroethylstarch in a 5:1 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. After removal of the WBC-poor plasma, the cells were resuspended in their own plasma, which were used for the freezing [25]. UCB was processed up to 48 h from delivery of the placenta.

Pre-cryopreservation samples were removed after removal of the WBC-poor plasma for colony-forming unit granulocyte–macrophage (CFU-GM) assay.

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).

Addition of disaccharides and bioantioxidants was always at the same concentration, 60 mmol/L and 100 μg/mL respectively. However, different concentrations of Me_2SO (2.5%, 5% and 10% (v/v)) were added to the cryovials in accordance with the intended concentrations (Table 1). The solutions were then refrigerated to 4 °C before addition of the cells, because the Me_2SO is toxic to cells at room temperature.

WBC-rich concentrates from two umbilical cord blood were pooled. Samples were divided in two groups:

First, 5% (v/v) Me_2SO (control), 5% (v/v) Me_2SO + trehalose, 5% (v/v) Me_2SO + sucrose, 5% (v/v) Me_2SO + ascorbic acid or catalase, 5% Me_2SO (v/v) + trehalose + ascorbic acid or catalase, 5% (v/v) Me_2SO + sucrose + ascorbic acid or catalase.

Second, 2.5% (v/v) Me_2SO (control), 2.5% (v/v) Me_2SO + trehalose, 2.5% (v/v) Me_2SO + sucrose, 2.5% (v/v) Me_2SO + ascorbic acid or catalase, 2.5% (v/v) Me_2SO + trehalose + ascorbic acid or catalase, 2.5% (v/v) Me_2SO + sucrose + ascorbic acid or catalase.

Table 1
Preparation of different cryoprotectant solutions.

Solution	Me_2SO (%v/v)	Sucrose (mmol/L)	Trehalose (mmol/L)	Acid ascorbic or catalase (μg/mL)
1	0	0	0	0
2	10	0	0	0
3	5	0	0	0
4	5	60	0	0
5	5	0	60	0
6	5	0	0	100
7	5	60	0	100
8	5	0	60	100
9	2.5	0	0	0
10	2.5	60	0	0
11	2.5	0	60	0
12	2.5	0	0	100
13	2.5	60	0	100
14	2.5	0	60	100

Two solutions, one of 10% (v/v) Me_2SO (concentration currently used in transplant centers) and one without cryoprotectant, were tested as well.

The choice of the concentration of disaccharides (60 mmol/L) was based on our previous studies [36] and the concentration of ascorbic acid (100 μg/mL) was based on literature [27].

In all the groups, 5×10^6 cells in 100 μL were transferred to 1-mL cryovials containing each cryoprotectant solution, and the cryovials were immediately frozen in accordance with the pre-established program of 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 3 weeks before thawing and further analysis. The cryopreservation of UCB cells, using the bioantioxidants, was an independent experiment, so analysis was therefore done separately.

Samples were thawed by rapidly immersing the cryovials in a 37 °C water bath, and then divided for each different analysis: the number of CD45+/CD34+ and CD45+/CD34+/CD123+ cells were measured by flow cytometry analysis, the hematopoietic progenitor content was accessed by a colony-forming units (CFU) assay and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay.

Flow cytometry analysis

CD34+ and CD34+/CD123+ 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. [23]. In accordance with this method, 2×10^6 cells/mL were incubated with anti-CD34PE (8G12 clone), anti-CD45FITC (2D1clone) and anti-CD123PerCP-Cy5.5 (7G3). Anti-CD123PerCP-Cy5.5 (7G3) is a mouse IgG2a monoclonal antibody that directly binds to the α-subunit of the human IL-3 receptor (BD Biosciences, San Jose, CA, USA).

The percentage of CD34+ and CD34+/CD123+ cells were determined in a FACSCAN (BD Biosciences, San Jose, CA, USA) with initial gating on CD45+ cells. To avoid damage to cells due to Me_2SO treatment, all post-thawed samples were washed with 9 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 duplicate.

Colony-forming unit (CFU) assays

CFU assays were carried out as described by Limaye and Kale [27]. After washing, 5×10^4 cells/mL (with each different cryoprotectant solution) 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) and rHu-SCF (20 ng/mL) without rHuEPO. The plates were incubated along with sterile water-containing plates for 14 days in 5% laboratory CO_2 in 300/3000 Series Incubators (Revco, Ashille, Ohio, USA). The colonies were then scored under an inverted microscope (Leica DMIL, Leica Microsystems, AG, Wetzlar, Germany). This test was also performed with pre-freeze samples for later comparison.

MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide)

MTT assay provides a simple method for the determination of cell viability using standard microplate absorbance readers. The MTT assay is based on the phenomenon that metabolic active cells cleave the yellow tetrazolium salt MTT to form purple formazan crystal [30]. The post-washed 1×10^5 cells/mL solutions (with each different cryoprotectant concentration) were plated (in triplicate) with 10 μL MTT solution to each well, incubated at 37 °C for 4 h and then measured for absorbance on an ELISA plate reader with a test wavelength of 570 nm.

Statistical analysis

All data was analysed with a statistical software package GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA, USA, (www.graphpad.com)). Two-way ANOVA test was used for comparisons of groups of more than two samples and Bonferroni post-tests. Results with *p*-values less than 0.05 were considered significant.

Results

Flow cytometry analysis

All results using flow cytometry were compared with their post-thawing controls.

CD45low/CD34high – samples cryopreserved with ascorbic acid (Fig. 1)

There was a statistically significant increase in the percentage of CD34+ cells from the post-thawed cryopreserved UCB with

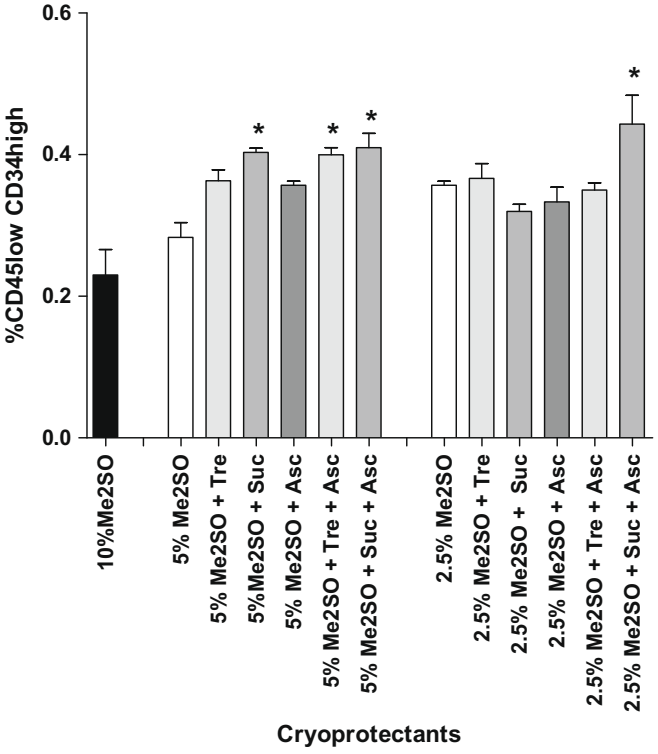

Fig. 1. Flow cytometric analysis of post-thawed UCB cells. The detection of the cells expressing both the CD34+ antigen and the CD45+ panleukocyte antigen can be used to measure the subpopulations of hematopoietic stem and progenitor cells. A single-platform flow cytometric method is available for rapid 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 concentration of Me_2SO (2.5%, 5% and 10% (v/v)) and, the same concentration of trehalose or sucrose (60 mmol/L) with/without ascorbic acid (100 μg/mL).

concentrations of 5% (v/v) Me_2SO + sucrose, 5% (v/v) Me_2SO + trehalose and ascorbic acid, and 5% (v/v) Me_2SO + sucrose and ascorbic acid, when compared with 5% (v/v) Me_2SO alone ($p < 0.05$). For samples frozen with 2.5% (v/v) Me_2SO, only those that were frozen in the presence of sucrose + ascorbic acid had a significant difference ($p < 0.05$).

The remaining solutions tested showed equal preservation of their cell sample compared to their control.

CD45+/CD34+/CD123+ – samples cryopreserved with ascorbic acid (Fig. 2)

The CD123 is constitutively expressed on committed hematopoietic stem/progenitor cells where it mediates proliferation and differentiation [20].

Samples cryopreserved in the presence of ascorbic acid + disaccharides showed a statistically significant increase in preservation CD123+ cells from both groups compared with their controls $p < 0.05$, except the solution with 2.5% (v/v) Me_2SO, disaccharides and ascorbic acid, that only had the tendency to this result.

CD45low/CD34high – samples cryopreserved with catalase (Fig. 3)

These results were gathered from experiments performed independently of those previous. All solutions tested achieved the same standard of preservation of receptors in both groups, compared with their controls. Several solutions showed a statistically significant increase in receptor preservation as mentioned in Fig. 3.

Flow cytometric analysis showed that there was a greater preservation of CD34+ cells in solutions containing both disaccharides and catalase. These results were statistically different compared to their controls (5% and 2.5% (v/v) Me_2SO), $p < 0.05$.

CD45+/CD34+/CD123+ – samples cryopreserved with catalase (Fig. 4)

Catalase showed a greater cryopreservation of CD123+ cells compared to its control but performed even greater cryopreservation of CD123+ cells when present with disaccharides, especially in solution with 2.5% (v/v) Me_2SO and sucrose (Fig. 4). However, solutions with 5% (v/v) Me_2SO and sucrose or trehalose showed results to equal their controls.

MTT assay

The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolic active cells [30]. The formazan is then solubilized, and the concentration determined by optical density at 570 nm. The result is a sensitive assay with a colorimetric signal proportional to the cell number. The MTT method of quantitation is as sensitive as the standard manual method, is especially useful for large numbers of samples, and requires no specialized laboratory equipment. In this assay, all solutions tested were statistically greater than the samples frozen without cryoprotectant, as expected ($p < 0.001$). The solutions tested were equal or better when compared with their controls (2.5% and 5% (v/v) Me_2SO). A statistically significant ($p < 0.05$) increase in cell viability in solutions containing disaccharides and catalase with 2.5% and 5% (v/v) Me_2SO was detected (Fig. 5).

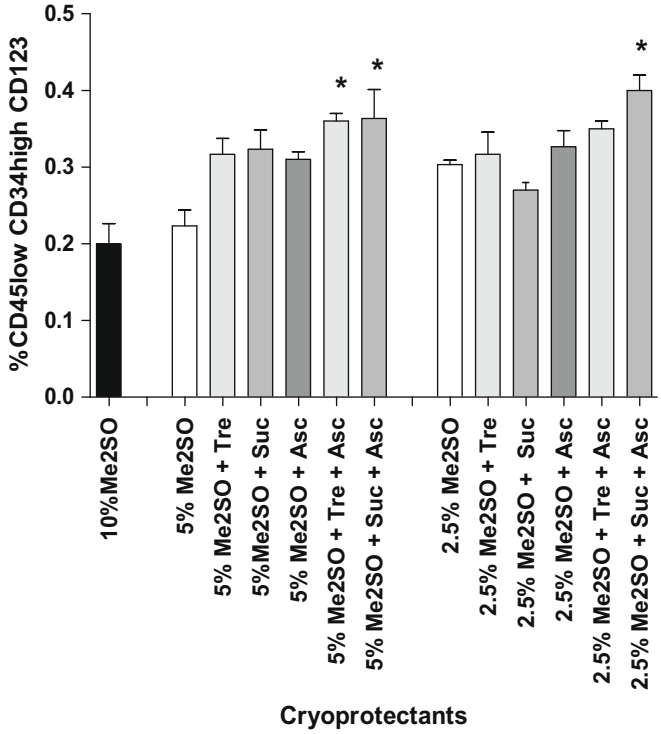

Fig. 2. Flow cytometric analysis of post-thawed UCB cells cryopreserved with different concentration of Me_2SO (2.5%, 5% and 10% (v/v)) and, the same concentration of trehalose or sucrose (60 mmol/L) with/without ascorbic acid (100 μg/mL). The percentage of cells CD34+/CD123+ were determined in a FACSCAN (BD Biosciences, San Jose, CA, USA) with initial gating on CD45+ cells. The IL-3 plays a critical functional role in hematopoiesis and exerts its effects through a specific IL-3 receptor (IL-3R or CD123).

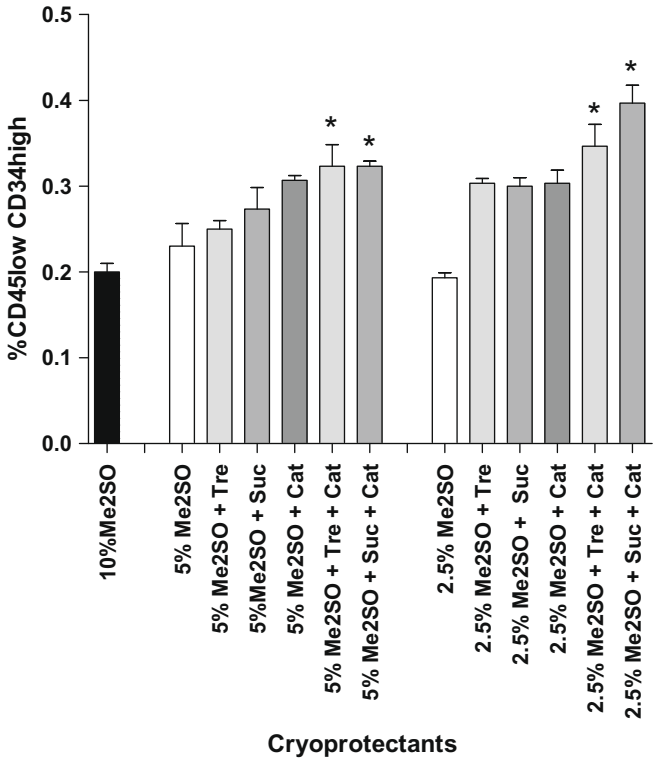

Fig. 3. Flow cytometric analysis of post-thawed UCB cells. The detection of the cells expressing both the CD34+ antigen and the CD45+ panleukocyte antigen can be used to measure the subpopulations of hematopoietic stem and progenitor cells. A single-platform flow cytometric method is available for rapid 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 concentration of Me_2SO (2.5%, 5% and 10% (v/v)) and, the same concentration of trehalose or sucrose (60 mmol/L) with/without catalase (100 μg/mL).

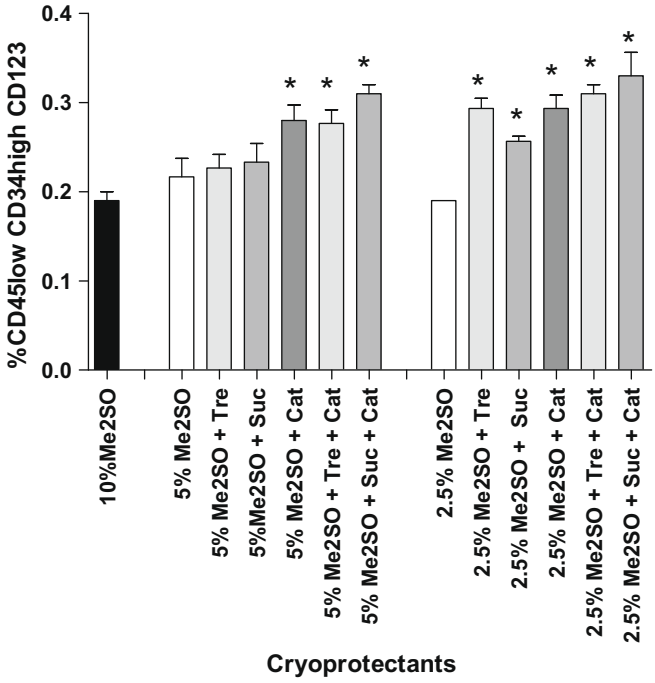

Fig. 4. Flow cytometric analysis of post-thawed UCB cells cryopreserved with different concentration of Me_2SO (2.5%, 5% and 10% (v/v)) and, the same concentration of trehalose or sucrose (60 mmol/L) with/without catalase (100 μg/mL). The percentage of cells CD34+/CD123+ were determined in a FACSCAN (BD Biosciences, San Jose, CA, USA) with initial gating on CD45+ cells. The IL-3 plays a critical functional role in hematopoiesis and exerts its effects through a specific IL-3 receptor (IL-3R or CD123).

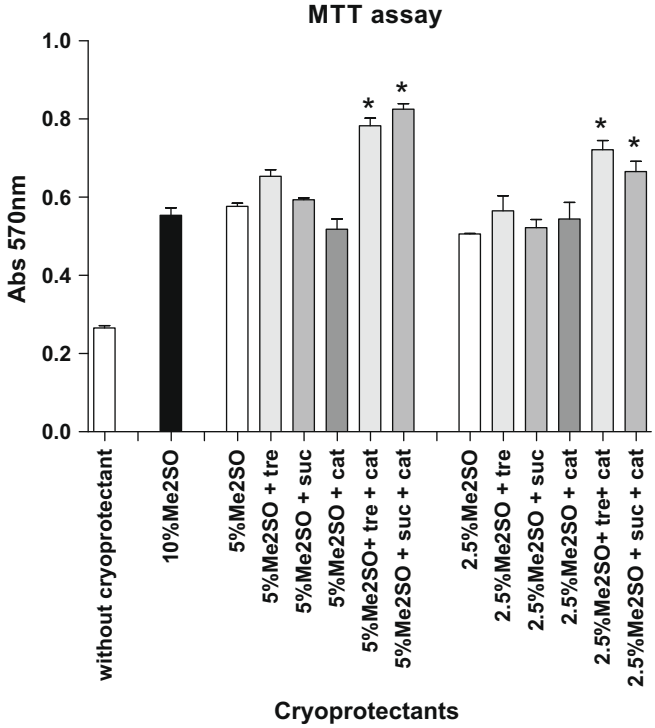

Fig. 5. MTT assay of post-thawed UCB cells cryopreserved with different compositions of Me_2SO, trehalose, sucrose and catalase. The cleavage of the tetrazolium salt MTT into a blue coloured product (formazan) by the mitochondrial enzyme succinate-dehydrogenase is potentially very useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to number of cells present.

Ascorbic acid has a high reactivity with MTT so solutions containing this substance were not considered.

Colony-forming unit (CFU) assays

All values of CFUs were expressed as a percentage of pre-cryopreservation values of the source UCB, which the samples were extracted from (Tables 2 and 3).

In our assessment of the percentage recovery of the clonogenic potential of cells frozen in the presence of ascorbic acid, we found that cells containing the antioxidant, ascorbic acid, performed no significant difference in improving the ability to form clones (Table 2).

However, when we analysed the cells frozen with catalase, we saw, especially on group two (2.5% (v/v) Me_2SO), a trend toward improved maintenance of clonogenic capacity of cells that were frozen in the presence of antioxidant and disaccharides (Table 3).

With the addition of catalase, total CFU increased approximately 5% compared with the solution that contained only 2.5% (v/v) Me_2SO + trehalose and increased 11% compared with the solution containing 2.5% (v/v) Me_2SO + sucrose. For specific CFU (CFU-GM and CFU-G) of group two samples, the increase was even higher. These solutions showed a statistically significant difference from their controls (2.5% (v/v) Me_2SO), which did not occur with cells frozen without the presence of catalase (Table 3).

With the addition of catalase the CFU-GM increased by 30% compared with the CFU-GM with 2.5% (v/v) Me_2SO + trehalose and by 35% when the samples were frozen with 2.5% (v/v) Me_2SO + sucrose alone. The addition of catalase increased the CFU-G by 8% compared with the CFU-G of 2.5% (v/v) Me_2SO + trehalose and by 100% when the samples were frozen with 2.5% (v/v) Me_2SO + sucrose (Table 3).

This cryoprotectant effect of catalase cannot be observed in CFU-M.

Discussion and conclusion

UCB is a particularly important source of stem cells for ethnic groups that are poorly represented in current bone marrow transplantation registries. One issue in UCB banking is that there is only one opportunity to obtain the samples. Overall, it is limited in volume and cell number [29].

Table 2
Clonogenic capacities of post-thawed CB HSC cryopreserved with ascorbic acid.

	CFU	CFU-GM	CFU-G	CFU-M
Without cryoprotectant	14.8	9.5	14.5	17.9
10% Me_2SO	49.5	28.6	38.6	66.3
5% Me_2SO (control)	61.8	62.3	56.8	63.7
5% Me_2SO + trehalose	81.3	71.8	85.9*	84.5
5% Me_2SO + sucrose	76.3	76.4	90.5*	87.2
5% Me_2SO + ascorbic acid	69.2	57.7	71.4	74.3
5% Me_2SO + trehalose + ascorbic acid	60.5	62.3	61.8	59.1
5% Me_2SO + sucrose + ascorbic acid	43.3	19.1	71.4	53.8
2.5% Me_2SO (control)	29.1*	37.7	37.7	44.3*
2.5% Me_2SO + trehalose	59.3*	63.6	62.7*	82.1*
2.5% Me_2SO + sucrose	62.9*	28.2	80.5*	81.9*
2.5% Me_2SO + ascorbic acid	40.8	14.1	33.2*	58.8
2.5% Me_2SO + trehalose + ascorbic acid	55.7	38.6	67.7*	58.9
2.5% Me_2SO + sucrose + ascorbic acid	52.0	34.1	43.6	66.6

Values of CFU, CFU-GM, CFU-G and CFU-M are expressed as a percentage of pre-cryopreservation values of same UCB. $p < 0.05$ (*).

Table 2 CFU assay of UCB cells cryopreserved with compositions of Me_2SO (2.5%, 5% and 10%). Trehalose or sucrose (60 mmol/L) with/without ascorbic acid (100 μg/mL). An in vitro assay system for pluripotent hemopoietic stem cells that possess the capacity to self-renew and provide differentiated progenies has been pursued by investigators for several years.

Table 3
Clonogenic capacities of post-thawed CB HSC cryopreserved with catalase.

	CFU	CFU-GM	CFU-G	CFU-M
Without cryoprotectant	17.3	10.6	7.4	21.5
10% Me_2SO	47.3	31.7	50.1	49.4
5% Me_2SO	54.1	39.4	48.0	59.4
5% Me_2SO + trehalose	60.0	47.2	44.6	68.3
5% Me_2SO + sucrose	66.1*	45.0*	55.6	76.0
5% Me_2SO + catalase	80.6*	71.1*	69.9*	83.7
5% Me_2SO + trehalose + catalase	63.2	60.8*	75.4*	68.6
5% Me_2SO + sucrose + catalase	54.8	66.7*	65.4	44.3
2.5% Me_2SO	44.2*	34.7*	32.7	57.4*
2.5% Me_2SO + trehalose	71.4*	79.4*	77.8	82.2*
2.5% Me_2SO + sucrose	70.5*	46.7	42.0	90.0*
2.5% Me_2SO + catalase	73.3*	50.0*	35.3*	95.0*
2.5% Me_2SO + trehalose + catalase	74.6*	77.5*	84.7*	71.0
2.5% Me_2SO + sucrose + catalase	78.6*	63.3*	84.7*	76.1

Values of CFU, CFU-GM, CFU-G and CFU-M are expressed as a percentage of pre-cryopreservation values of same UCB. $p < 0.05$ (*).
Table 3 CFU assay of UCB cells cryopreserved with compositions of Me_2SO (2.5%, 5% and 10%). Trehalose or sucrose (60 mmol/L) with /without ascorbic acid (100 μg/mL). An in vitro assay system for pluripotent hemopoietic stem cells that possess the capacity to self-renew and provide differentiated progenies has been pursued by investigators for several years.

Since the first human cord blood transplant was performed 20 years ago [15], cord blood banks have been established around the world. For the maintenance of these samples, an appropriate cryopreservation protocol should occur. When cells are exposed to the ice formation that accompanies low temperatures, they are subjected to major changes in their intrinsic physical structure, resulting in cell injury. This is basic principle of cryobiology. Cryoprotective agents (such as Me_2SO) can be added to cell suspensions to mitigate cryoinjury and increase cell survival following freezing and thawing [19]. But several side effects have been described during infusion of Me_2SO, such as hypotension, nausea, headache and others more severe [41]. Optimization of freezing protocols to get improved recovery has been our goal. The use of membrane stabilizers and bioantioxidants has been shown to offer cryoprotection.

In this paper, we used two disaccharides (trehalose and sucrose) and two antioxidants (catalase and ascorbic acid) in combination with low concentrations of Me_2SO in the cryopreservation of stem cells from UBC and analysed the effects of freezing, 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.

Studies of natural systems that survive extreme environmental stress, such as freezing and desiccation, have shown that one of the adaptive mechanisms is the accumulation of sugars, such as trehalose [9,10]. The mechanism of trehalose protection is an active area of research that includes the interaction of sugars with plasma membranes [11], the role of glassy state [17], the effects on cell osmotic responses [7], and the unique physico-chemical properties of trehalose [34]. Regardless of the mechanism of action, relatively low concentrations of non-toxic trehalose have been reported to act as an effective cryoprotective in a wide variety of biological systems [1,5,13].

Another hypothesis proposed of injury during hypothermia and freezing is the formation of oxygen-free radicals, which have been implicated as a potential cause of viability loss of cells during or just after freezing [26]. The stability of free radicals which is low in systems of high water content is considerably increased under low moisture and subfreezing conditions [22]. If not properly scavenged, it results in oxidative damage such as lipid peroxidation, protein oxidation, and DNA damage. A cell's major defense against free-radical-mediated damage includes antioxidants such as ascorbic acid, α-tocopheryl acetate (vitamin E), and reduced glutathione and enzymes such as superoxide dismutase, catalase and peroxidases [6].

In this study, the addition of antioxidants, mainly catalase, and disaccharides in freezing solutions at low concentrations of Me_2SO, improved preservation of CD34+ cells post-thawed, compared with the control solution. The CD34 antigen is stage specific and identifies the cells in their earliest stages of hematopoietic differentiation [4]. The CD34+ population, therefore, contains progenitor cells committed to the myeloid, lymphoid, and erythroid lineages, as well as the primitive stem cells capable of long-term reconstitution. This population of CD34+ cells contains all of the colony-forming cells and the long-term culture-initiating cells.

By flow cytometry, we found that interleukin-3 receptor (CD123) expression was also very well preserved, when using low concentrations of Me_2SO, disaccharides and catalase. The CD123 is constitutively expressed on committed hematopoietic stem/progenitor cells where it mediates proliferation and differentiation, and because of this, the preservation of this receptor during cryopreservation is very important. Different levels of CD123 expression have been reported on CD34+ cells at different stages of differentiation: low expression on CD34+ cells identifies the more primitive compartment, while the maturing progenitors have high surface levels of CD123 [20].

The cytometry data was confirmed by the results of functional test (CFU), which showed that the optimal preservation of cells with clonogenic capacity and their receptors were in solutions containing catalase.

Through the cleavage of MTT to a blue formazan derivative by living cells, it was also indicated that frozen cells in the presence of catalase, are more viable after thawing. Limaye and colleagues showed that antioxidants like catalase, α-tocopheryl, and ascorbic acid – when used singularly as additives in conventional freezing medium – improve protection of mouse bone marrow cells and adult human bone marrow [26].

In the present study, analysis of the total number of CFU, CFU-GM and CFU-G, in the different cryoprotectant solutions, showed once again that solutions present with catalase better preserved the clonogenic potential of cells.

Samples frozen with Me_2SO + a disaccharide showed a significant difference in cryopreservation outcome compared to the control. However, the addition of catalase to solutions with Me_2SO + a disaccharide, further improved this already significant difference. We hypothesize that this is further indication of catalase's ability to reduce the reactive oxygen species. Limaye and colleagues showed that a combination of trehalose and catalase in conventional freezing medium helps in preserving human hematopoietic cells isolated from cord blood and fetal liver. The results obtained showed the combination of trehalose and catalase was improved preservation better than those obtained when the two additives were used separately [27]. Other studies using these combinations were performed, as Sasnoor and colleagues showed that trehalose and catalase were effective in preserving LTC (long-term culture) forming ability, surface molecule expression [38], and in vitro adhesion and chemotaxis [39] of frozen human hematopoietic cells.

Previously, we have reported the benefits of using trehalose and sucrose as additives to freezing medium containing low concentration of Me_2SO for freezing of hematopoietic stem cells of cord blood [36]. One of our goals in the present study was to see whether with the addition of bioantioxidant, helped to preserve the functionality of cells.

The mechanism of action of the two antioxidants in scavenging free radicals is different. Briefly, ascorbic acid is an electron donor, which neutralizes the reactive oxygen species, whereas the catalase breaks down hydrogen peroxide into H_2O and O_2. In this study, only catalase was found to be very effective and resulted in better protection of UBC cells. Peroxide radicals cause oxidative damage such as lipid peroxidation, protein oxidation, and DNA damage to cells, and perhaps the presence of exogenous catalase protects

these cells by scavenging peroxide. The use of these antioxidants under clinical situations can be recommended only after toxicity testing of i.v. infusion of the antioxidants. The protective effect of the two antioxidants needs to be further confirmed by in vitro assays such as long-term culture-initiating cells, high proliferative potential colony-forming cells, and in vivo animal experiments to study engraftment kinetics, and also by extending the storage period.

Taken together our results indicate that effective scavenging of reactive oxygen species and stabilization of membrane leading to reduction in cell deaths during freezing and thawing, underlies the better engraftment of hematopoietic stem cells of umbilical cord blood that were frozen with additives. Moreover, the development of a Me_2SO-free method of cryopreservation might improve the safety of hematopoietic cell transplantation by both, reducing the side effects for the patient and by avoiding toxic effects on the cryoprotected cells.

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