

# Effect of trehalose on cryopreservation of pure peripheral blood stem cells

DANIELA MARTINETTI<sup>1</sup>, CRISTINA COLAROSSO<sup>2</sup>, SIMONA BUCCHERI<sup>1,3</sup>,  
GABRIELLA DENTI<sup>1</sup>, LORENZO MEMEO<sup>1,2</sup> and LUISA VICARI<sup>1</sup>

<sup>1</sup>Cell Biology Unit, IOM Ricerca Srl, Viagrande, I-95029 Catania; <sup>2</sup>Department of Experimental Oncology, Mediterranean Institute of Oncology, Viagrande, I-95029 Catania; <sup>3</sup>Department of Laboratory Medicine and Advanced Biotechnologies, Fondazione Ri.MED, Regenerative Medicine and Biomedical Technologies Unit, IRCCS-ISMETT, I- 90133 Palermo, Italy

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**Abstract.** Stem cells are an important tool for the study of hematopoiesis. Despite developments in cryopreservation, post-thaw cell death remains a considerable problem. Cryopreservation protocol should limit cell damage due to freezing and ensure the recovery of the functional cell characteristics after thawing. Thus, the use of cryoprotectants is essential. In particular, the efficacy of trehalose has been reported for clinical purposes in blood stem cells. The aim of the current study was to establish an efficient method for biological research based on the use of trehalose, to cryopreserve pure peripheral blood stem cells. The efficacy of trehalose was assessed *in vitro* and the cell viability was evaluated. The data indicate that trehalose improves cell survival after thawing compared with the standard freezing procedure. These findings could suggest the potential for future trehalose application for research purposes in cell cryopreservation.

## Introduction

Stem cells in biological research provide a significant source of information and, thus, contribute to the development of novel therapeutic strategies. In particular, the ability to cultivate stem cells and human hematopoietic progenitor cells *in vitro* is the fundamental basis for investigating hematopoiesis. An improved understanding of the mechanisms that regulate cell proliferation and differentiation during the stages of hematopoiesis would further elucidate the molecular characteristics of diseases (which are characterized by excessive expansion or a functional defect of certain immature blood components), and facilitate the identification of substances that are able to specifically protect healthy cells from the action of cytotoxic drugs.

Hematopoietic stem and progenitor cells (HSPCs) may be isolated from peripheral blood (PB), bone marrow (BM) or umbilical cord blood (CB) (1).

Although significant improvements to cell cryopreservation procedures have been achieved (2), the improvement of current cryopreservation protocols that lead to a high mortality rate after thawing for the crystal formations that arise during freezing is a primary goal. Specifically, fast cooling forms intracellular ice crystals, which results in cell destruction and slow cooling forms ice crystals in the extracellular space, with consequent cellular dehydration. Selection of a cryoprotectant, as well as a suitable freezing rate serves to protect cells from these adverse effects (3,4).

Cryoprotectants are divided into two classes: Penetrating and nonpenetrating (5). The penetrating cryoprotectants include glycerol and 1,2-propanediol and dimethyl sulfoxide (Me<sub>2</sub>SO); the latter is commonly used for HSPC cryopreservation (6). The non penetrating cryoprotectants comprise polyvinyl pyrrolidone, trehalose, fructose, sucrose and glucose.

Trehalose is a non-toxic disaccharide of glucose that preserves the structural integrity of the cells during freezing and thawing (7). Specifically, trehalose is found in numerous organisms, such as nematodes and yeasts, which are capable of surviving during freezing and drying (8,9). Previous studies have used this disaccharide for cryopreservation of human cells, such as platelets (10), red blood cells (11), sperm (12), oocytes (13), pancreatic islets (14) and fetal skin (15). Furthermore, the use of an alternative protocol with trehalose for clinical applications to cryopreserve stem cells obtained from CB and bone marrow (BM) has previously been reported in the literature (16,17) and in a study by Scheinkönig *et al* (18) from mobilized PB stem cells (PBSCs). Conversely, in the current study, trehalose was administered, only for research purposes, to cryopreserve pure HSPCs, and not mobilized HSPCs, that were isolated from the PB of healthy individuals.

## Materials and methods

**Cytokines, antibodies and chemicals.** Ficoll-Hypaque (density, 1.077 g/ml) was purchased from Pharmacia Biotech, Uppsala, Sweden. A CD34 MultiSort kit human, midiMACS separator, MACS multistand, LS columns, and anti-CD34

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**Correspondence to:** Dr Lorenzo Memeo, Department of Experimental Oncology, Mediterranean Institute of Oncology, 7 Via Penninazzo, Viagrande, I-95029 Catania, Italy  
E-mail: lorenzo.memeo@grupposamed.com

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fluorescein isothiocyanate (FITC), human (clone: AC136; cat. no. 130-081-001), anti-CD61 PE, human (clone: Y2/51; cat. no. 130-081-501), mouse IgG2a-FITC isotype control (cat. no. 130-091-837) and mouse IgG1-PE isotype control (cat. no. 130-092-212) antibodies (all dilutions, 1:10) were purchased from Miltenyi Biotec, Inc. (Auburn, CA, USA); BIT 9500 Serum Substitute (Stem cell Technologies, Inc., Vancouver, BC, Canada); Gibco Iscove's Modified Dulbecco's Medium (IMDM) was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and recombinant human TPO was from PeproTech, Inc. (Rocky Hill, NJ, USA). Me<sub>2</sub>SO, D-(+)-Trehalose dihydrate, catalase from bovine liver, lipoprotein, low density lipoproteins (LDLs) from human plasma, trypan blue and May-Grünwald-Giemsa were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nalgene® Mr. Frosty® Cryo 1°C Freezing Containers were purchased from Thermo Fisher Scientific, Inc.

**Purification and characterization of CD34<sup>+</sup> cells.** Hematopoietic stem and progenitor cells (HSPCs) were obtained from the PB of 80 healthy donors supplied by IOM SPA (Viagrande, Italy) under an approved Institutional Review Board protocol (project ID code: 454\_1; 6 February 2008, IOM Institutional Review Board, Viagrande, Italy) and subsequent to obtaining informed consent.

Briefly, mononuclear cells (MNCs) were isolated from PB (20-30 ml) by Ficoll-Hypaque. After recovery of the cell suspension, 150-300x10<sup>6</sup> MNCs were magnetically labeled with CD34 Micro Beads and loaded onto a MACS® Column, which was placed in the magnetic field of the midi MACS separator. The CD34<sup>+</sup> cells are retained within the column and unlabeled cells run through. After the column was removed from the magnetic field, the magnetically retained CD34<sup>+</sup> cells were eluted as the positively selected cell fraction. The purity was evaluated by flow cytometry of the FITC-labeled anti-CD34 FITC antibody (dilution, 1:10) according to the manufacturer's instructions. Cells were analyzed by FACSCalibur (BD Biosciences) using BD CellQuest™ Pro (version 6.0; BD Biosciences) software for fluorescence intensity analysis. The CD34<sup>+</sup> morphology was evaluated by May-Grünwald-Giemsa staining. Cells were cytocentrifuged onto glass slides, stained with May-Grünwald-Giemsa and observed by light microscopy; the percentage of CD34<sup>+</sup> cells ranged from 89 to 98%.

#### Freezing media preparation for cryopreservation

**Freezing media preparation for cryopreservation of human CD34<sup>+</sup> cells with different trehalose concentrations or the classical method.** Freezing media were freshly made with different D-(+)-trehalose concentrations (1.25, 1.0.6 or 0.3 M) or with a medium containing 90% fetal bovine serum (FBS) and 10% Me<sub>2</sub>SO. Dilutions were prepared using serum-free medium (IMDM) from a 2.5-M trehalose stock solution in IMDM. The freezing media and cryogenic vials (NalgeNunc International, Penfield, NY, USA) were cooled prior to use.

**Freezing media preparation for cryopreservation of human CD34<sup>+</sup> cells with different cryoprotectants for short- and long-term cryopreservation.** Five freezing media were freshly

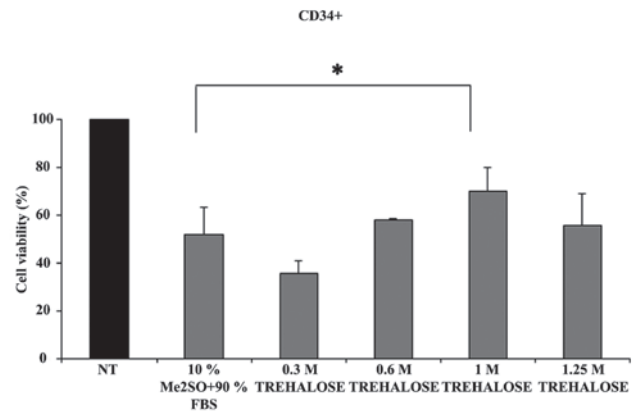


Figure 1. Percentage of cell viability measured after thawing of the CD34<sup>+</sup> cells previously frozen with different concentrations of trehalose or according to the classical method. CD34<sup>+</sup> cells were frozen with: 0.3, 0.6, 1 and 1.25M trehalose or with 10% Me<sub>2</sub>SO + 90% FBS. The viability after thawing was evaluated by Trypan Blue dye exclusion assay. Data were analyzed using Student's t-test and are expressed as the mean ± standard deviation of five separate experiments. \*P<0.05. Cell viability (%) = number of recovered cells after thawing x100/number of non-cryopreserved cells (NT, 100% viability). Me<sub>2</sub>SO, dimethyl sulfate; FBS, fetal bovine serum.

prepared as follows: 10% Me<sub>2</sub>SO + 90% FBS; 1M trehalose; 1M trehalose + 10% Me<sub>2</sub>SO; 1M trehalose + 100 µg/ml catalase; and 1 M trehalose + 100 µg/ml catalase + 10% Me<sub>2</sub>SO. The freezing media and cryogenic vials were cooled prior to use. The trehalose and catalase were freshly prepared in IMDM.

**Cryopreservation of human CD34<sup>+</sup> cells.** The optimal concentration of CD34<sup>+</sup> cells (200,000) were frozen in cryogenic vials in a Nalgene® controlled-rate freezing container (NalgeNunc International) by a gradual addition of freezing medium in a final volume of 200 µl. The freezing container contained 100% isopropyl alcohol that provided a 1°C/min cooling rate when stored at -80°C in a controlled freezer for the initial freezing cycle prior to placement in a liquid nitrogen freezer (-196°C) until final use. The CD34<sup>+</sup> cells were cryopreserved for a short (20 days) and longer (3 months) duration.

**Thawing of human CD34<sup>+</sup> cells.** Frozen cells were rapidly thawed in a water bath at 37°C, gently shaken and removed when the ice crystals dissolved. Subsequently cell viability and the ability to differentiate into megakaryocytes (MKs) were evaluated. Cell viability was assessed using the dye exclusion test with trypan blue and counts were performed in triplicate. Percentages of viable cells after thawing were normalized to non-cryopreserved cells (NT, 100% viability) using the following equation: Cell viability (%) = number of recovered cells after thawing x100/number of non-cryopreserved cells.

**MK unilineage cultures.** MK differentiation was induced by growing CD34<sup>+</sup> for 14 days in BIT 9500 Serum Substitute in the presence of human LDL and 100 ng/ml thrombopoietin. Cells were incubated at 37°C for 14 days in a fully humidified atmosphere of 5% CO<sub>2</sub>. The MK differentiation stage was evaluated after thawing by flow cytometry of typical anti-CD61<sup>+</sup> PE antibody (dilution, 1:10).

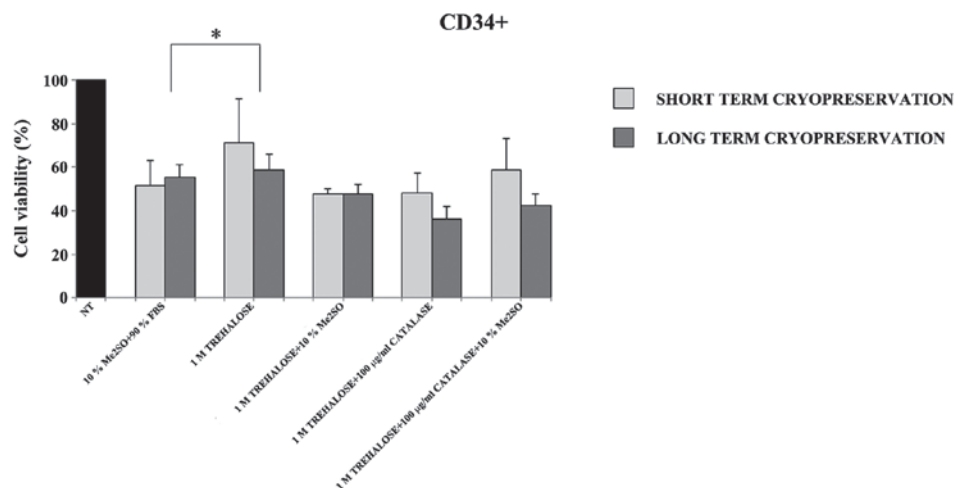


Figure 2. Percentage of cell viability measured after thawing of CD34<sup>+</sup> cells previously frozen with different cryoprotectants at short (20 days) and long (3 months) durations of cryopreservation. CD34<sup>+</sup> were frozen with: 10% Me<sub>2</sub>SO+90% FBS; 1M trehalose; 1M trehalose + 10% Me<sub>2</sub>SO, 1M trehalose + 100 µg/ml catalase; 1 M trehalose + 100 µg/ml catalase + 10% Me<sub>2</sub>SO. The viability after thawing was evaluated by trypan blue dye exclusion assay. Data were analyzed using Student's t-test and are expressed as the mean ± standard deviation of five separate experiments. \*P<0.05. Cell viability (%) = number of recovered cells after thawing x100/number of non-cryopreserved cells (NT, 100% viability). Me<sub>2</sub>SO, dimethyl sulfate; FBS, fetal bovine serum.

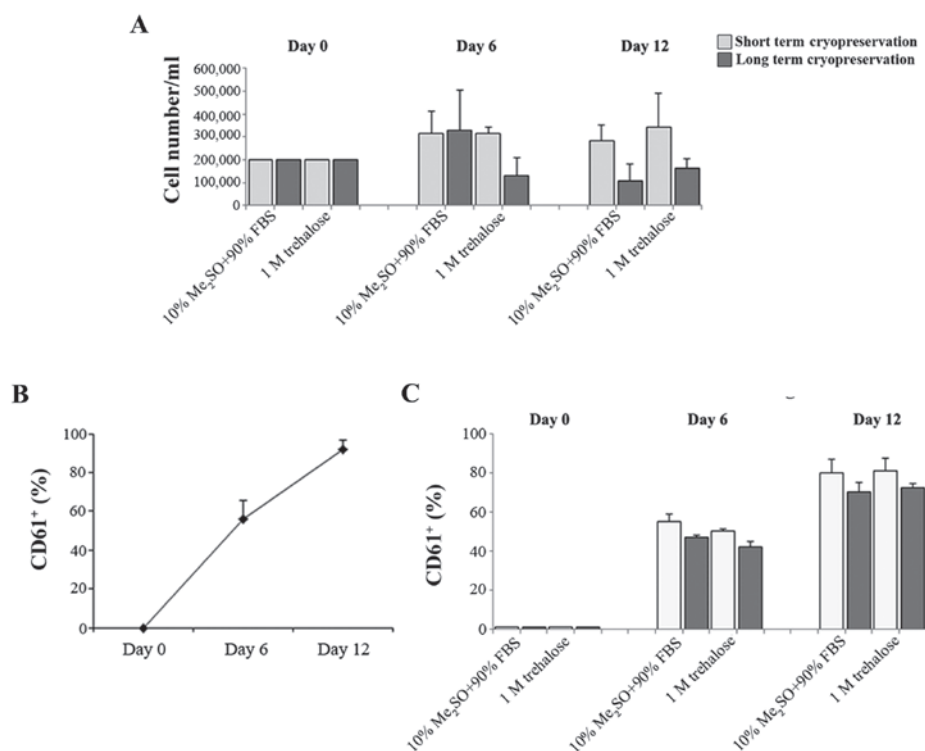


Figure 3. Ability of the CD34<sup>+</sup> to generate MKs subsequent to thawing. (A) Number of MKs obtained from CD34<sup>+</sup> cells previously frozen with 1M trehalose or according to the classical method, following short (20 days) and long (3 months) durations of cryopreservation. The evaluation was performed by trypan blue dye exclusion assay on the day of thawing (day 0), and after 6 and 12 days of MK differentiation. Data are expressed as the mean ± standard deviation of three separate experiments. (B and C) Percentage of CD61<sup>+</sup> at different stages of MK differentiation. MKs were obtained from (B) fresh CD34<sup>+</sup> or (C) after thawing. Cells were previously cryopreserved with 1M trehalose or 10% Me<sub>2</sub>SO + 90% FBS at short and long durations of cryopreservation. The evaluation was performed by flow cytometry of typical anti-CD61<sup>+</sup> PE antibody on the day of thawing (day 0), and after 6 and 12 days of MK differentiation. Data are expressed as the mean ± standard deviation of four separate experiments. MK, megakaryocyte; FBS, fetal bovine serum.

**Statistical analysis.** Data were analyzed using Student's t-test, and the results were expressed as the mean ± standard deviation of separate experiments. P<0.05 was considered to indicate a statistically significant difference.

## Results

The aim of the current study was to develop an effective cryopreservation technique for pure HSPCs obtained from PB to

replace the standard method that uses Me<sub>2</sub>SO. HSPCs were isolated from healthy individuals after acquiring informed consent. The expression levels of the CD34 surface stem marker was evaluated by flow cytometry obtaining a percentage of CD34<sup>+</sup> cells between 89 and 98%. Morphological observations subsequent to May-Grünwald-Giemsa staining correlated with the flow cytometry data.

As the quality of the cryopreserved cells is also cell concentration-dependent (19), human CD34<sup>+</sup> were initially frozen at different cell concentrations (100,000, 150,000 and 200,000) with a medium containing FBS and Me<sub>2</sub>SO. Subsequent to thawing, cell viability was assessed using the dye exclusion test with trypan blue. The results revealed that the optimal concentration to cryopreserve CD34<sup>+</sup> was 200,000 (data not shown).

Subsequently, the effect of cryopreservation on CD34<sup>+</sup> cells treated with four different trehalose concentrations compared with Me<sub>2</sub>SO was evaluated (Fig. 1). The findings indicated that freezing with 1M trehalose provided improved cryoprotection to CD34<sup>+</sup> when compared with the standard freezing procedure using Me<sub>2</sub>SO.

Furthermore, as it has been reported in previous studies (20,21) that freezing with trehalose in combination with an antioxidant (catalase) or with Me<sub>2</sub>SO could improve cell viability, CD34<sup>+</sup> cells were cryopreserved for a short (20 days) and long (3 months) duration, with a medium containing different combinations of catalase, Me<sub>2</sub>SO and trehalose. The current data indicate that, after thawing, the number and viability of the CD34<sup>+</sup> cells was higher in all the samples cryopreserved with only 1M trehalose (Fig. 2).

The hematopoietic stem cell is also responsible for the formation of differentiated cells in the blood that must be continually replaced through the proliferation and differentiation of immature forms. Megakaryopoiesis is a process of hematopoietic differentiation previously investigated by the current authors (22). Therefore, the ability of the fresh CD34<sup>+</sup> cells and those obtained after thawing to proliferate and differentiate in MKs was compared. These cells were previously cryopreserved with 1M trehalose or Me<sub>2</sub>SO for short (20 days) and long (3 months) cryopreservation durations. MK differentiation was induced by growing CD34<sup>+</sup> for 14 days and evaluated by flow cytometry of the typical anti-CD61<sup>+</sup> antibody. CD34<sup>+</sup> cells that were previously frozen with trehalose retained the ability to form MK subsequent to thawing (Fig. 3A). In addition the percentage of CD61<sup>+</sup> cells obtained after thawing using the two different methods (Fig. 3C) is similar to that of MK produced from fresh CD34<sup>+</sup> cells (Fig. 3B).

## Discussion

Hematopoietic stem cells enable investigation of the underlying mechanisms of hematopoietic development for the ability to differentiate into different cell types. In addition, they may be used for drug development and as model systems to evaluate certain diseases for which there are no effective treatments currently available.

Cryopreservation allows cell preservation overlong periods by exposure to extremely low temperatures. Although this procedure causes suspension of metabolic reactions, during the freezing and thawing phases considerable physical changes

occur, which can be detrimental to cell survival. As a result of cryopreservation, there is marked loss of cell viability and function. Therefore, the addition of a cryoprotectant to the cells is important. Among the cryoprotectants used, trehalose, which is a sugar that is utilized in cell cryopreservation, is significant (10-15). Trehalose is utilized in many fields, such as in the pharmaceutical industry (contained in various commercially available therapeutic products), the food industry (in confectionary products) and in the cosmetics industry (for example, in bath oils, creams and lotions) (23). Furthermore, the efficacy of trehalose in clinical cryopreservation of human stem cells from cord blood (24-26), bone marrow and mobilized PBSCs (18) has been widely demonstrated. Conversely, in the present study trehalose was used only for research purposes to cryopreserve pure and not mobilized PBSCs, in order to identify a more efficient protocol, which avoids cell loss that occurs during the normal course of cryopreservation. It was identified that 1M trehalose resulted in improved cryoprotection to CD34<sup>+</sup> when compared with the standard freezing procedure that uses Me<sub>2</sub>SO. Furthermore, freezing with trehalose in combination with catalase or Me<sub>2</sub>SO did not increase cell viability. In addition, maintenance of the viability and ability of CD34<sup>+</sup> cells to differentiate into MKs after thawing at short and long-term of cryopreservation was observed.

In conclusion the data indicate that the use of trehalose allows a greater number of hematopoietic stem cells to be obtained after thawing. The basis of a suitable cryopreservation protocol was established in the current study. However, future investigations are required to assess the repeatability on a commercial scale.

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