

Pentaisomaltose, an Alternative to DMSO. Engraftment of Cryopreserved Human CD34⁺ Cells in Immunodeficient NSG Mice

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Abstract

Hematopoietic stem cell transplantation often involves the cryopreservation of stem cell products. Currently, the standard cryoprotective agent (CPA) is dimethyl sulfoxide (DMSO), which is known to cause concentration-related toxicity and side effects when administered to patients. Based on promising *in vitro* data from our previous study using pentaisomaltose (a 1 kDa subfraction of Dextran I) as an alternative to DMSO for cryopreservation of hematopoietic progenitor cells (HPCs) from apheresis products, we proceeded to a preclinical model and compared the two CPAs with respect to engraftment of human hematopoietic stem and progenitor cells (HSPCs) in the immunodeficient NSG mouse model. Human HPCs from apheresis products were cryopreserved with either pentaisomaltose or DMSO, and the following outcomes were measured: (1) the post-thaw recovery of cryopreserved cells and clonogenic potential of CD34⁺ cells and (2) hematopoietic engraftment in NSG mice. We found that recovery and colony-forming cells data were comparable between pentaisomaltose and DMSO. The engraftment data revealed comparable human CD45⁺ levels in peripheral blood at 8 weeks and bone marrow at 16 weeks post transplantation. Additionally, the frequencies of CD34⁺CD38^{low/negative} and myeloid/lymphoid cells in the bone marrow were comparable. We here demonstrated that long-term engrafting HSPCs were well preserved in pentaisomaltose and comparable to cells cryopreserved with DMSO. Although a clinical trial is necessary to translate these results into human use, the present data represent an important step toward the replacement of DMSO with a non-toxic alternative.

Keywords

pentaisomaltose, DMSO, cryopreservation, CD34⁺ cells, hematopoietic progenitor cells, cryoprotective agent

Introduction

Hematopoietic stem cell transplantation involves intravenous infusion of autologous or allogeneic stem cells to reestablish bone marrow (BM) function after a conditioning regimen with chemotherapy and possibly irradiation. For the autologous setting and the use of cord blood units, cryopreservation is a mandatory procedure. Dimethyl sulfoxide (DMSO) is the gold-standard cryoprotective agent (CPA). However, both minor and more severe adverse reactions are associated with the infusion of thawed cell products containing DMSO^{1–4}. In addition, the unpleasant smell of DMSO and its metabolites affects both patients and working environments.

DMSO has also been reported to exert toxic effects on cells, reduce the expression of key factors related to stemness, and induce epigenetic changes^{1,5–11}. Therefore, there has been an increasing demand from health care

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professionals and authorities to develop non-toxic cryopreservation alternatives devoid of DMSO and xeno-additives.

In a previous study, we tested pentaisomaltose *in vitro*, for cryopreservation of hematopoietic progenitor cells (HPCs) from apheresis products. Pentaisomaltose is a 1-kDa subfraction of Dextran 1 which is approved for clinical use, and as an extracellular cryoprotectant it is less likely than DMSO to interact with the intracellular molecules. The results demonstrated that cells cryopreserved in a freezing medium that contained pentaisomaltose exhibited post-thaw recovery of viable CD34⁺ cells, a distribution of CD34⁺ subpopulations, and colony-forming potential which were comparable to HPCs cryopreserved using DMSO¹². The next step before possibly conducting a clinical trial is to investigate if the hematopoietic stem and progenitor cells (HSPCs) maintain their potential *in vivo* in a preclinical model. We therefore compared the engraftment of HSPCs cryopreserved in DMSO or pentaisomaltose in a humanized immunodeficient NSG mouse model.

Materials and Methods

Five patients (three females and two males, aged 20–59 years) were enrolled in the study and donated the cells used for both the *in vitro* and *in vivo* experiments.

Patient Samples

All patients scheduled for peripheral blood stem cell collection at Rigshospitalet, Copenhagen University Hospital, Denmark were invited to participate in the study. The patients included were diagnosed with multiple myeloma or malignant lymphoma. Peripheral blood stem cells were mobilized and collected by apheresis according to a previously described procedure¹².

Cryopreservation of Peripheral Blood Stem Cell Products

Cryopreservation was performed no later than 24 hours from the time of leukapheresis according to the previously published protocol¹². Cells were cryopreserved in DMSO and pentaisomaltose in parallel. Briefly, cryomedia containing 32% pentaisomaltose (Pharmacosmos A/S, Denmark) or 20% DMSO (WAK-Chemie, Germany) in 4% human albumin (CSL Behring, Denmark), and 2 IE/mL heparin (Amgros I/S, Denmark), was prepared. Cryomedia was mixed 1:1 with HPC(A) products in cryovials (final cell concentration of 50–95 × 10⁶ total nucleated cells (TNCs) per/mL) (Nunc, Thermo Scientific, Denmark) and cryopreserved in a controlled rate freezer (profile: start temp. 4°C, –1°C/min to 0°C, –2°C/min to –45°C and –5°C/min to –100°C, Kryo 560-16, Planer PLC, UK). The cryovials were transferred to liquid nitrogen (–190°C) and stored until use. Samples were cryopreserved for 2–9 months before they were thawed and used for the engraftment assay.

Recovery of Viable Cells in Cryopreserved Apheresis Products

The quantification of viable TNCs, mononucleated cells (MNCs), granulocytes, and CD34⁺ cells for both pre-freeze and post-thaw samples was performed according to a previously published protocol¹². Briefly, cryopreserved samples were thawed, diluted 1:10 and stained with FITC-labeled anti-CD45 and PE-labeled anti-CD34 antibodies, and 7AAD was used as a live/dead marker (Stem Kit, Beckman Coulter, Denmark). Samples were analyzed by flow cytometry. The recovery values of TNCs, MNCs, granulocytes, and CD34⁺ cells were calculated as the number of viable cells post-thaw relative to pre-freeze.

Colony-Forming Cell Assay

The colony-forming cells (CFCs) potential was assessed according to a previously published protocol¹². Briefly, 1 × 10⁴ thawed mononuclear cells were seeded in media (MethoCult H4034 Optimum, Stemcell Technologies, France) supporting differentiation and growth of myeloid progenitors. The total number of erythroid (BFU-E and CFU-E) and non-erythroid (CF-G, CFU-M, CFU-GM, and CFU-GEMM) colonies were counted manually after 14–16 days of culture. In addition, the total numbers of cells in the colonies were quantified by flow cytometry following 14–16 days of culture, using ECD-labeled anti-CD45 (Beckman Coulter, Denmark) and APC-labeled anti-CD71 (Miltenyi, Sweden) antibodies.

Preparation of Samples for Transplantation into NSG Mice

Samples were thawed in a 37°C water bath, and Pulmozyme (final concentration: 125 Units/mL, Roche, Poland) and MgCl₂ (final Mg²⁺ concentration: 2.5 mM) were immediately added after thawing to reduce DNA-mediated aggregation. The samples were rehydrated in a buffer containing Dulbecco's PBS, 2% human albumin (CSL Behring, Denmark) and 2 IE/mL heparin (Amgros I/S, Denmark) and incubated for 30 min at room temperature. The samples were centrifuged for 5 min at 300 × g, resuspended in buffer, and the CD34⁺ cells were purified by CD34⁺ MACS selection (Miltenyi Biotec, Sweden), according to the manufacturer's instructions. CD34-labeled cells were eluted in Dulbecco's PBS and 2% FCS (Thermo Fisher Scientific, Sweden) and maintained on ice until transplantation.

Human Engraftment Assay

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NSG; Jackson Laboratory, USA) were sublethally irradiated (300 cGy) before transplantation. A total of 86 mice (12–16 weeks old) were divided into five groups. To avoid any possible influence of sex or age of the mice on the comparison

between the two CPAs, each group was further divided into two sex- and age-matched groups. Thus, each sample from one patient, cryopreserved in either pentaisomaltose or DMSO, was intravenously injected into the tail vein of 8–9 mice with 50×10^3 viable purified CD34⁺ cells per mouse. Xenogeneic NSG transplantations are associated with a high degree of mortality. Therefore, we used a high number of mice for each sample to ensure a representative estimation of the average engraftment value. The difference in the number of data points between patients reflects that several recipients died or were euthanized post transplantation due to poor physical condition. There was no significant difference in the overall survival of mice transplanted with samples cryopreserved in DMSO and PIM (28% versus 14%).

Peripheral blood (PB) was sampled from the tail vein at 8 weeks post transplantation and the levels of human CD45⁺ cells were measured. The mice were euthanized at 16 weeks post transplantation, and the BM (Left and right tibia, femur and iliac bones) was investigated for the presence of lymphoid and myeloid cells. The human cell contribution in the PB and BM of the NSG mice was assessed by an APC-labeled anti-human CD45 antibody (Biolegend, USA). The lymphoid/myeloid distribution was evaluated with PE-labeled anti-CD33 (BD Biosciences, Denmark), FITC-labeled anti-CD14 (Biolegend, USA), PE/Cy7-labeled anti-CD3 (eBioscience, USA), and BV605-labeled anti-CD19 (BD Biosciences, Denmark) antibodies. The frequency of CD34⁺CD38^{low/negative} cells was also evaluated by PE-labeled anti-CD38 (BD Biosciences, Denmark) and FITC-labeled anti-CD34 (Biolegend, USA) antibodies. The samples were analyzed on a flow cytometer (FACSCanto II, Becton Dickinson, Denmark), and the data were analyzed using the accompanying software (FACSDiva, Version 6.1.3, Becton Dickinson, Denmark).

Statistics

A paired *t*-test was used to analyze the recovery, CFC and engraftment data (mean engraftment values for each patient samples). Values are presented as mean \pm Standard deviation (SD). A *p*-value below 0.05 was considered significant. All statistical analyses were performed using GraphPad prism version 5.02 (GraphPad software Inc, CA, USA).

Results

Comparable Recovery of TNC, MNC, and CD34⁺ Cell and Colony-Forming Cell Potential but Increased Recovery of Granulocytes when using Pentaisomaltose instead of DMSO

To investigate the quality of the cell samples, we tested them *in vitro* by measuring the recovery of viable TNCs,

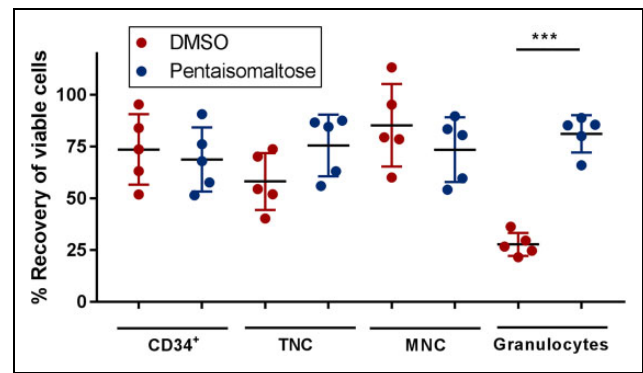


Fig 1. Recovery of TNCs, MNCs, granulocytes and CD34⁺ cells in cryopreserved apheresis samples. Recovery of TNCs, MNCs, granulocytes and CD34⁺ cells was measured by flow cytometry. We did not find any significant differences in recovery of TNCs, MNCs, or CD34⁺ cells, but the recovery of granulocytes was significantly higher when using pentaisomaltose compared to DMSO. Samples from 5 patients were used in the experiment.

MNCs, granulocytes and CD34⁺ cells and the CFC potential. The mean \pm SD recovery of total nuclear cells ($75.7\% \pm 14.9$ and $58.2\% \pm 13.8$), mononuclear cells ($73.9\% \pm 14.9$ and $85.4\% \pm 20.0$; *p* = 0.055), and viable CD34⁺ cells ($68.9\% \pm 15.5$ and $73.7\% \pm 17.2$) were not significantly different when using pentaisomaltose or DMSO, respectively. However, we found a significantly increased recovery of granulocytes ($81.3\% \pm 9.0$ and $27.8\% \pm 5.6$; *p* < 0.0001), when using pentaisomaltose compared with DMSO (Fig 1). In addition, in the CFC assay performed to assess the number of erythroid (CFU-E and BFU-E) and myeloid (CFU-G, CFU-M, CFU-GM and CFU-GEMM) HPCs and the total number of cells in the harvested colonies as a measure of proliferative potential following cryopreservation, both CPAs achieved similar results (table 1).

Comparable Engraftment of CD34⁺ Cells Cryopreserved in Pentaisomaltose or DMSO in the Immunodeficient NSG Mouse Model

To test *in vivo* how well the HSPCs were preserved using the two CPAs, we assessed their engraftment capacity in NSG mice (experimental setup depicted in Fig 2A). For these experiments, CD34⁺ cells were magnetically purified from the thawed apheresis samples to reduce the levels of contaminating xenoreactive T cells, and the cells were subsequently transplanted into NSG recipients (8–9 mice per group). We measured the engraftment levels in the transplanted NSG mice after 8 and 16 weeks and found no significant differences in the frequencies of human CD45⁺ cells between the samples cryopreserved in pentaisomaltose or DMSO from either 8-week PB (Fig 2B) or 16-week BM samples (Fig 2C). In patient 1 and 5 sufficiently high engraftment enabled assessment of

Table I. Colony-Forming Cell Potential of Cryopreserved Apheresis Products.

	DMSO		Pentaisomaltose	
	Mean number	Range	Mean number	Range
<i>Colonies (CFU-G, M, GM, GEMM and BFU-E)</i>				
Erythroid (CD45 ⁺ CD71 ⁺ and CD45 ⁻ CD71 ⁻)	13.9	8.8-18.5	11.9	7.7-18.5
Non-erythroid (CD45 ⁺ CD71 ⁻)	23.3	13.5-36.0	21.9	15.0-37.5
<i>Cells per well ($\times 10^6$)</i>				
Erythroid (CD45 ⁺ CD71 ⁺ and CD45 ⁻ CD71 ⁻)	1.75	0.82-2.54	2.13	1.56-2.96
Non-erythroid (CD45 ⁺ CD71 ⁻)	0.41	0.26-0.58	0.47	0.38-0.65

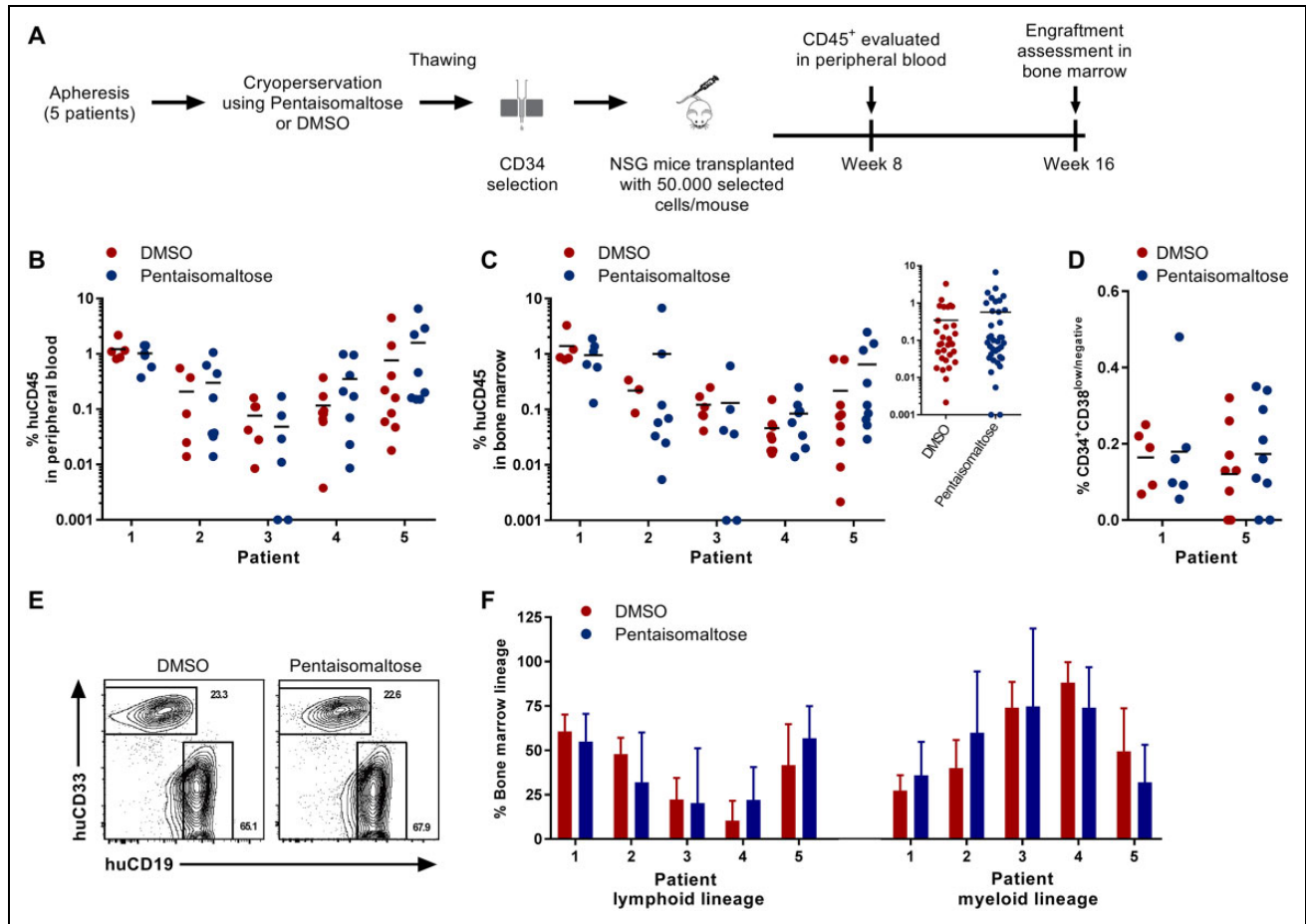


Fig 2. Engraftment of CD34⁺ cells cryopreserved in pentaisomaltose or DMSO-based freezing medium in NSG mice. (A) Experimental setup. CD34⁺ cells were positively selected from thawed HPC(A) samples. A total of 50×10^3 CD34⁺ cells were transplanted into sublethally irradiated (300 cGy) NSG mice. Engraftment was evaluated by flow cytometry, which was calculated as the frequency of human CD45⁺ cells in (B) peripheral blood at 8 weeks post transplantation and (C) bone marrow at 16 weeks post transplantation (The superscript figure shows all pooled mice). (D) Frequency of CD34⁺CD38^{low/neg} cells within the human CD45⁺ population. (E) Representative flow cytometry plots showing the CD33 and CD19 gating. (F) Lymphoid and myeloid potential measured by the expression of CD33/CD14 (myeloid cells) and CD19/CD3 (lymphoid cells) in the bone marrow of NSG recipients at 16 weeks post transplantation. Samples from 5 patients were used.

CD34⁺CD38^{low/negative} cell frequencies, and also in this analysis similar levels were observed for DMSO and pentaisomaltose (Fig 2D). Furthermore, human CD45⁺ cells in BM samples showed comparable frequencies of lymphoid and myeloid cells (Fig 2F).

Discussion

Our group has previously demonstrated in vitro that cryopreservation of HPC(A) using pentaisomaltose provides recovery of CD34⁺ cells, CD34⁺ subpopulations, and CFCs equivalent to that of using DMSO as a CPA¹².

In the current *in vivo* study, we demonstrated successful hematopoietic engraftment of HSPCs cryopreserved in low-molecular weight pentaisomaltose in the NSG mouse model. Also, the *in vitro* results in the present study demonstrated comparable recovery of HSPC, erythroid, and myeloid progenitors and proliferative potential after cryopreservation with either DMSO or pentaisomaltose. These data confirmed our previously published *in vitro* results¹² and showed that the samples were of reliable quality for the engraftment study.

Consistent with the *in vitro* results, the frequency of human CD45⁺ cells and immature CD34⁺CD38^{low/negative} cells in BM was comparable using the two CPAs. This suggests comparable engraftment, and that HSPCs with long-term engraftment capacity are equally well preserved with both CPAs.

Engraftment levels may vary considerably in the model of NSG mice, depending on, for example, the source of CD34⁺ cells (umbilical cord blood, BM or mobilized PB) and whether the cells are fresh or cryopreserved^{13,14}. Most importantly, engraftment levels were not significantly different when using pentaisomaltose or DMSO.

An attractive feature of the NSG mouse model is the development of multilineage hematopoietic cells after the transplantation of human HSPCs. Thus, we assessed the lympho-myeloid distribution in the BM at 16 weeks post transplantation and detected similar levels of lymphoid and myeloid cells for each patient when using pentaisomaltose or DMSO. We observed some variation in the overall engraftment and the lymphoid/myeloid distribution between the patients, which, in addition to general inter-individual variation, may reflect differences in disease state and/or treatment, including the use of medical products that affect specific cell populations, for example anti-CD20 (rituximab). Of note, all the corresponding HPC(A) products were previously engrafted in the autologous setting in the clinic.

In the present study, we used post-thaw CD34⁺-selected cells from cryopreserved samples, in contrast to the unselected HPC(A) products that are most often used in the clinic. However, in a pilot study, we tested the use of unselected grafts and found that in both PB and BM more than 90% of human CD45⁺ cells were T cells, indicating peripheral proliferation, probably resulting from a xenogeneic graft-versus-host-like response (data not shown). Therefore, post-thaw selection of CD34⁺ cells from HPC(A) was performed before transplantation of the NSG mice, a setup well-established^{13–15}. The cell dose of 50×10^3 viable CD34⁺ cells/mouse corresponds to approx. 2.5×10^6 cells/kg body weight, well within the normal range used in the clinic.

With respect to possible adverse effects of administration of 16 g of pentaisomaltose (a subfraction of Dextran 1) corresponding to a 100 mL cryopreserved apheresis product, clinical investigations with Dextran 1 have shown that a single dose of 24 g as well as repeated doses of 5×12 g within an hour do not induce clinical significant adverse effects¹⁶. Also, Dextran 1 is quickly excreted by the kidneys

(the half-life in serum is 30 min) and potential adverse reactions are therefore expected to be of short duration¹⁶.

One of the putative mechanisms for adverse infusion reactions that have been associated with high levels of granulocytes is the breakdown of granulocytes during thawing of DMSO-preserved apheresis products^{2,17,18}, due to low osmotic tolerance and release of intracellular contents¹⁹. Interestingly, we found the recovery of viable granulocytes to be higher when pentaisomaltose was used as a CPA compared with DMSO. This might be explained by the assumed extracellular effect of pentaisomaltose, which does not lead to an increase in intracellular osmo-active molecules, as is the case with DMSO. Therefore, a reduced risk of excessive osmotic cell swelling and an improved recovery of granulocytes could be an additional potential benefit of using pentaisomaltose instead of DMSO.

In conclusion, our present and previously published data collectively suggest that mobilized HSPCs cryopreserved in DMSO- or pentaisomaltose-based freezing media are comparable in regard to cell survival and clonogenic potential *in vitro*, as well as long-term engraftment potential in a preclinical *in vivo* model. Although a clinical trial is necessary to translate these results into human use, the present data represent an important step toward the replacement of DMSO with a non-toxic alternative.

Author Contribution

J.D.S., M.S.T. and J.L. designed research, performed experiments, analyzed data, and wrote the manuscript. L.M-F., M.B.H., J.S.G. and P.A. designed the study and wrote the manuscript and E.K.H. and A. F-N. designed and directed the study, analyzed data, and wrote the manuscript.

Ethical Approval

The study was approved by the local research ethical committee of the Copenhagen Capital region (Protocol nr: H-4-2013-140). All animal experiments were approved by the Lund/Malmö ethical committee for animal research.

Statement of Human and Animal Rights

The study was conducted in accordance with the Helsinki declaration.

Statement of Informed Consent

The criteria for inclusion of patients were written consent.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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