Low-molecular-weight carbohydrate Pentaisomaltose may replace dimethyl sulfoxide as a safer cryoprotectant for cryopreservation of peripheral blood stem cells

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BACKGROUND: Cryopreserved hematopoietic stem cell products are widely used for certain hematologic malignancies. Dimethyl sulfoxide (DMSO) is the most widely used cryoprotective agent (CPA) today, but due to indications of cellular toxicity, changes of the cellular epigenetic state, and patient-related side effects, there is an increasing demand for DMSO-free alternatives. We therefore investigated whether Pentaisomaltose (PIM), a low-molecular-weight carbohydrate (1 kDa), can be used for cryopreservation of peripheral blood stem cells, more specifically hematopoietic progenitor cell apheresis (HPC(A)) product.

STUDY DESIGN AND METHODS: We cryopreserved patient or donor HPC(A) products using 10% DMSO or 16% PIM and quantified the recovery of CD34+ cells and CD34+ subpopulations by multicolor flow cytometry. In addition, we compared the frequency of HPCs after DMSO and PIM cryopreservation using the colony-forming cells (CFCs) assay.

RESULTS: The mean CD34+ cell recovery was 56.3 ± 23.7% (11.4%-97.3%) and 58.2 ± 10.0% (45.7%-76.9%) for 10% DMSO and 16% PIM, respectively. The distribution of CD34+ cell subpopulations was similar when comparing DMSO or PIM as CPA. CFC assay showed mean colony numbers of 70.7 ± 25.4 (range, 37.8-115.5) and 67.7 ± 15.7 (range, 48-86) for 10% DMSO and 16% PIM, respectively.

CONCLUSION: Our findings demonstrate that PIM cryopreservation of HPC(A) products provides recovery of CD34+ cells, CD34+ subpopulations, and CFCs similar to that of DMSO cryopreservation and therefore may have the potential to be used for cryopreservation of peripheral blood stem cells.

Cryopreservation of cells and tissues has been of great interest to the scientific community since 1949, when Polge and coworkers[1] reported the cryoprotective effect of glycerol. Today, cryopreservation is an important tool both in the clinic and in research. In the clinical setting,

ABBREVIATIONS: 7AAD = 7-aminoactinomycin; CFC(s) = colony-forming cell(s); CPA = cryoprotective agent; HPC(A) = hematopoietic progenitor cell (apheresis); HSC(s) = hematopoietic stem cell(s); MPP(s) = multipotent progenitor(s); PIM = Pentaisomaltose.

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hematopoietic stem cells (HSCs) are collected and cryopreserved for later use for autologous transplantation, which is widely used for certain hematologic malignancies, and cord blood transplantation, which has been increasingly used for allogeneic HSC transplantation during the past decades.[2,3]

The use of a cryoprotective agent (CPA) is crucial to maintain cell viability after cryopreservation and subsequent thawing. The current standard CPA is dimethyl sulfoxide (DMSO). However, DMSO can exert toxic effects on cells depending on the concentration, temperature, and exposure time during both the prefreeze and the postthaw periods.[4-6]

Additionally, adverse reactions related to DMSO are frequent and well documented. Minor reactions are reported in up to 67.4% of patients and include allergic and gastrointestinal symptoms as well as hypo- or hypertension.[7,8] More severe adverse reactions, such as cardiac or respiratory arrest, neurotoxicity, and epileptic seizures, were reported in 1.4% of transplanted patients in a study of 97 EBMT transplant centers.[6,9] The necessity of infusing the newly thawed products ice cold may contribute to these reactions. A significant feature of DMSO and its metabolic derivatives is a strong and unpleasant smell, which is a nuisance for staff, patients, and copatients.

At a cellular level, DMSO has been shown to down regulate the expression of stem cell–specific transcription factors such as Oct-4, Sox-2, Nanog, and Rex-1 in human embryonic stem cells.[10,11] DMSO also had an impact on the epigenetic state of mouse embryoid bodies, induced genomewide epigenetic changes in a murine preosteoblastic cell line, and stimulated the methyltransferase activity of de novo DNA methyltransferase 3a.[12-14] Due to these drawbacks of using DMSO as a CPA, there is an increasing demand for DMSO- and xeno-free nontoxic cryopreservation alternatives.

CPAs are classified as either cell permeating or nonpermeating. DMSO belongs to the former category and is capable of displacing and binding to water molecules within the cell, thereby preventing the formation of intracellular ice crystals leading to cell damage.[15] Nonpermeating CPAs range from relatively small molecules such as sucrose and trehalose to large molecules such as hydroxyethyl starch (HES) and dextran 40. Several of these molecules have shown promising results and have facilitated the use of lower DMSO concentrations.[16,17] However, a cell-permeating CPA such as DMSO seems to be inevitably necessary to achieve a sufficient cryoprotective effect.

In line with the use of HES and high-molecular-weight dextrans for cryopreservation, we previously performed a pilot screening of low-molecular-weight carbohydrates and derivatives. This screening indicated that the 1-kDa carbohydrate Pentaisomaltose (PIM) may act alone as a nontoxic CPA. PIM is a subfraction of dextran 1 and complies with the specification in the monographs for dextran 1 in the European and US Pharmacopoeias. These findings led to the current study, where we present in vitro data demonstrating the successful cryopreservation of hematopoietic progenitor cell apheresis (HPC(A)) products using PIM alone as a CPA.

MATERIALS AND METHODS

Patient and donor samples

All patients and allogeneic donors scheduled for peripheral blood stem cell collection at Rigshospitalet, Copenhagen University Hospital, Denmark, from Autumn 2013 until Spring 2015 were invited to participate in the study. The patients were primarily diagnosed with malignant lymphoma or multiple myeloma, and the allogeneic donors were all HLA-matched related donors. Peripheral blood stem cells were mobilized from patients using chemotherapy combined with granulocyte–colony-stimulating factor (G-CSF, Neupogen Novum, Amgen), whereas donors received G-CSF alone. The apheresis procedures were performed on an apheresis system (Spectra Optia, Terumo BCT) using the MNC program. The criteria for inclusion of patients and donors were informed consent and a number of collected CD34+ cells exceeding the clinical need. The exclusion criteria were withdrawal of the informed consent or the absence of an excess of collected CD34+ cells. The study was approved by the local ethical committee of the Copenhagen Capital region (Protocol H-4-2013-140) and was conducted in accordance with the Helsinki Declaration.

Cryopreservation of HPC(A) products

Cryopreservation was performed no later than 24 hours from the time of leukapheresis, and the products were kept in gas-permeable bags at 4°C if stored overnight. Cryomedia were prepared a few hours before use and mixed 1:1 with the apheresis product samples. Cryomedia consisted of: 1) 20% DMSO (WAK-Chemie) or PIM (Pharmacosmos A/S) at the concentrations specified, 2) 4% human albumin (CSL Behring), and 3) 2 IE/mL heparin (Amgros I/S).

All handling steps of the cell material and cryomedia were performed on ice to reduce cell activity to a minimum. For cryopreservation, 0.5 mL of precooled apheresis product was transferred to a 1.8 mL cryovial (Nunc, Thermo Scientific); 0.5 mL of cryomedium was then slowly added. The samples were gently mixed by hand, incubated on ice for 15 minutes, and frozen using a controlled-rate freezer (Kryo 560-16, Planer PLC) utilizing gradient-based cooling (start temp. 4°C, −1°C/min drop to 0°C, −2°C/min drop to −45°C, and −5°C/min drop to −100°C), which is the current controlled-rate freezing profile used for cryopreservation of clinical products. The

samples were stored in a liquid nitrogen container (−190°C).

Recovery of viable CD34+ cells

Enumeration of viable CD34+ cells for both prefreeze and postthaw samples was performed by flow cytometry using a lysis–no-wash procedure and a single platform analysis. Briefly, the samples were thawed (cryopreserved samples only) in a 37°C water bath, diluted 1:10 in phosphate-buffered saline (PBS) plus 2% human albumin, and incubated with a monoclonal antibody (MoAb) cocktail containing anti-CD45-FITC, anti-CD34-PE, and 7-aminoactinomycin (7AAD; Stem-Kit, Beckman Coulter). Fluorospheres (Stem-Count, Stem-Kit, Beckman Coulter) were used for quantification according to the manufacturer's instructions. Samples and data were analyzed on a flow cytometer (Navios, Beckman Coulter) using the accompanying software (Cytometer 1.2, Navios). The recovery of viable CD34+ cells was calculated as the number of viable CD34+ cells/mL HPC(A) product after thaw, relative to the number before freeze with the postthaw samples corrected according to the twofold dilution in cryomedium.

Colony-forming cell assay

Colony-forming units (CFUs) were determined using medium (MethoCult H4034 Optimum, Stemcell Technologies) formulated to support the optimal growth of erythroid progenitors (CFU-E and BFU-E); granulocyte/macrophage progenitors (CFU-GM, CFU-M, CFU-G); and multipotent granulocyte, erythroid, macrophage, and megakaryocyte progenitors (CFU-GEMM) according to the manufacturer's guidelines. Briefly, 1×10^4 mononuclear cells were incubated in methylcellulose medium supplemented with recombinant human stem cell factor, GM-CSF, G-CSF, IL-3, and erythropoietin. The cells were seeded in ultralow-binding multiwell plates (Corning) in duplicate and incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. After 16 days of culture, the total numbers of colonies were counted manually using an inverted microscope (Carl Zeiss).

To evaluate and compare the CFU cells generated by the samples cryopreserved in either DMSO or PIM, we collected all colonies from each well, pooled well duplicates, and performed flow cytometric enumeration of CD45+ and CD71+ cells. Briefly, the wells were placed on ice for 10 minutes and harvested in ice-cold buffer (Dulbecco's PBS containing 2% human albumin; CSL Behring). The cells were centrifuged for 10 minutes at 400 × *g*, resuspended in buffer, and stained with anti-CD71 (APC) (Miltenyi) and anti-CD45 (ECD) (Beckman Coulter) MoAbs. Cell numbers were quantified using fluorescent beads (Beckman Coulter). Samples and data were analyzed on a flow cytometer (Navios, Beckman Coulter) using the accompanying software (Navios Cytometer 1.2).

CD34+ subpopulations

To determine the frequencies of CD34+ subpopulations, HPC(A) samples were incubated with a mixture of blocking antibodies to avoid nonspecific binding. Subsequently, we added a mixture of surface-specific MoAbs, including anti-CD34 (PE), anti-CD45RA (FITC), anti-CD38 (APC), anti-CD90 (BV510), anti-CD123 (BV421), anti-CD3 (PerCP), anti-CD11b (PerCP), anti-CD15 (PerCP), and anti-CD19 (PerCP). All antibodies were purchased from Biolegend (Nordic BioSite) and used according to the manufacturer's instructions. The cells were diluted with buffer and centrifuged for 5 minutes at 300 × *g*. The pellet was resuspended in buffer and analyzed on a flow cytometer (FACSCanto II, Becton Dickinson). Data were analyzed using the accompanying software (FACSDiva, Version 6.1.3, Becton Dickinson).

Statistical analysis

A paired t test was used to analyze the recovery and HSC subpopulation data. The Wilcoxon matched-pairs test was used for the analysis of CFU colony numbers. A paired t test was used to analyze CFU cell numbers. All statistical analyses were performed with computer software (GraphPad Prism, Version 5.02, GraphPad Software Inc.).

RESULTS

A total of 17 patients and one donor were included in the study. Samples from 14 patients were used to determine the optimal concentration and recovery. Samples from seven patients were used for the CFU assay. Four of these samples were also used for the analysis of colony cell numbers, surface markers, and CD34+ subpopulations.

Recovery of viable CD34+ cells using low-molecular-weight PIM is comparable to DMSO

To determine the optimal concentration of PIM for the cryopreservation of HPC(A) samples, we compared CD34+ cell recovery for four different PIM concentrations (8, 12, 16, and 20%) to 10% DMSO. The best recoveries were found with 16 or 20% PIM (data not shown), and further studies were performed using a concentration of 16% PIM for cryopreservation of samples. The mean ± SD recovery of viable CD34+ cells was similar for DMSO (56.3 ± 23.7%; range, 11.4–97.3) and 16% PIM (58.2 ± 10.0%; range: 45.7%–76.9%; Fig. 1; $p = 0.77$).

The recovery of CD34+ subpopulations shows the same distribution after cryopreservation with DMSO and PIM

The CD34+ cell population is heterogeneous and contains both multipotent HSCs and the more differentiated

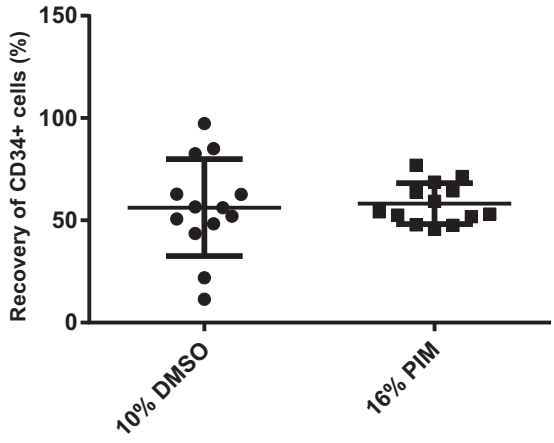

Fig. 1. Recovery of viable CD34+ cells from apheresis samples cryopreserved with 10% DMSO or 16% PIM (n = 12). The recovery of CD34+ cells after thawing is expressed as the percentage of the number of CD34+ cells before cryopreservation. All data points represent duplicate measurements.

HPCs. To obtain an indication of the hematopoietic potential of CD34+ cells cryopreserved with DMSO or PIM, we analyzed the relative distribution of viable subpopulations within the CD34+ population using flow cytometry (Fig. 2). The CD34 + CD38low/– gate contains multipotent HSCs and multipotent progenitors (MPPs) with the ability to provide long- and short-term engraftment after transplantation, respectively.[18] Because CD38 expression on CD34+ cells is a continuum rather than a dichotomous distribution, we set a gate for the DMSO cryopreserved cells to include 10% of the CD34+ cells with the lowest CD38 expression and used these specific gate settings for the samples cryopreserved with PIM. Thus, we could compare the relative recovery of viable CD34+ CD38low/– cells as well as the relative distribution of subpopulations within this gate (Fig. 2). As shown in Table 1, we found a similar recovery of CD34+ subpopulations after cryopreservation with 10% DMSO and 16% PIM, with no significant difference in the frequency of HSCs ($p = 0.086$).

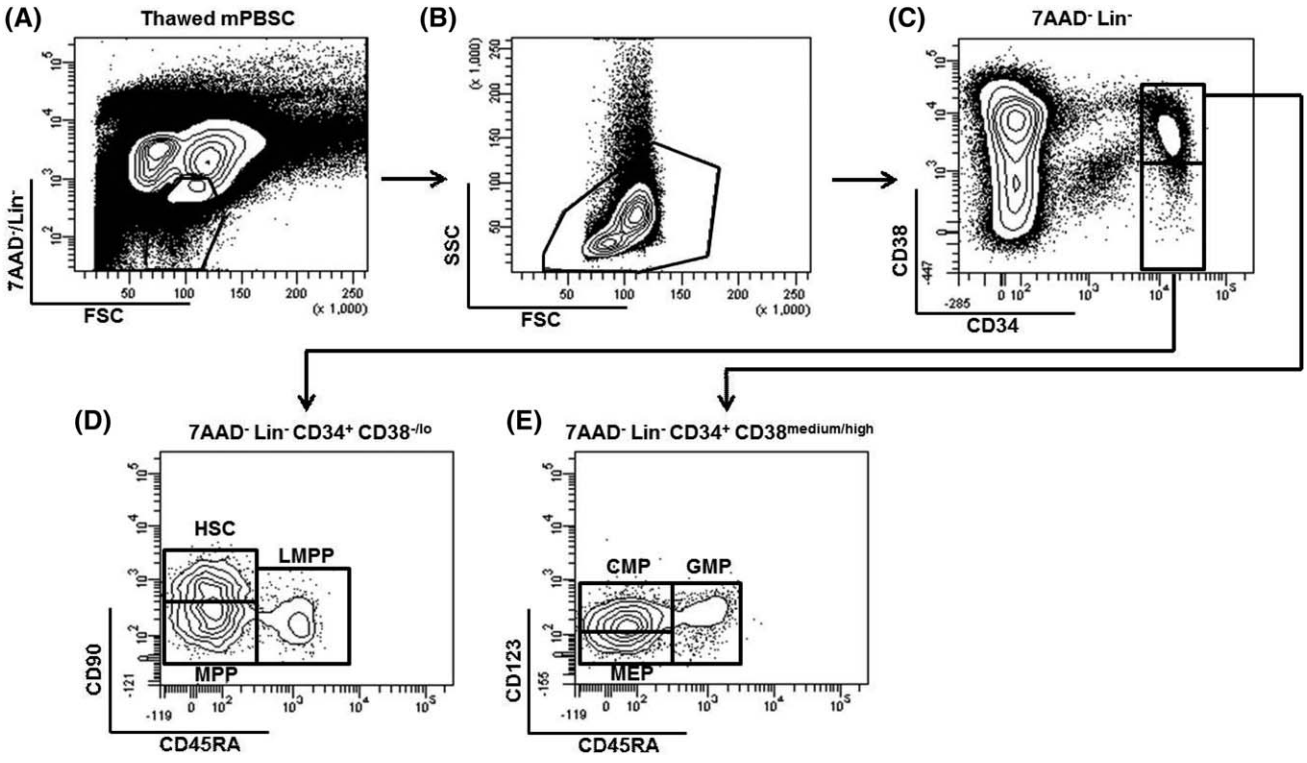

Fig. 2. Gating strategy for subpopulations in apheresis samples cryopreserved in DMSO or PIM. Subpopulations were analyzed using flow cytometry to detect the expression of CD34, CD38, CD90, CD45RA, and CD123 and the absence of the lineage markers CD3, CD11b, CD15, and CD19 (Lin–). (A) *Gating on* lineage-negative (Lin–) viable cells (7AAD–). (B) Back-gating of 7AAD–Lin– cells. (C) *Gated on* 7AAD–Lin– cells is used to distinguish between 7AAD–Lin–CD38medium/high and 7AAD–Lin–CD38low/– cells among the CD34+ cells (for details, see Results). (D) *Gated on* 7AAD–Lin–CD34+ CD38low/– cells. (E) *Gated on* 7AAD–Lin–CD34+ CD38medium/high cells. LMPP = lymphoid-primed multipotent progenitor cell; CMP = common myeloid progenitor; GMP = granulocyte macrophage progenitor; MEP = megakaryocyte erythroid progenitor. The data represent four patients.

TABLE 1. Distribution (%) of CD34+ subpopulations in apheresis products cryopreserved with DMSO or PIM (n = 4)

Subpopulations	10% DMSO	16% PIM
HSC	4.6 (3.1-6.7)	3.6 (1.4-5.5)
MPP	4.7 (2.6-6.5)	5.3 (3.9-6.4)
LMPP	0.2 (0-0.4)	0.4 (0-0.8)
CMP	41.6 (35.9-48.0)	41.8 (39.0-46.6)
GMP	13.5 (5.8-21.1)	17.5 (11.2-23.7)
MEP	35.3 (21.1-45.5)	31.4 (19.9-39.6)

CMP = common myeloid progenitor; GMP = granulocyte macrophage progenitor; LMPP = lymphoid-primed multipotent progenitor cell; MEP = megakaryocyte erythroid progenitor.

HPC(A) samples cryopreserved with PIM and DMSO show comparable frequencies of colony-forming cells

Colony-forming cell (CFC) assays were set up to investigate whether HPCs in cryopreserved HPC(A) samples retained their hematopoietic potential and were able to produce cells of the myeloid and erythroid lineages. As depicted in Fig. 3A, samples cryopreserved with both 10% DMSO and 16% PIM contained viable HPCs with the potential to produce colonies of the CFU-E and BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM lineages.

In addition, we found no significant difference ($p = 0.68$) in CFC numbers after 16 days of culture with the aforementioned specific growth medium (Fig. 3B). The mean CFC numbers using 10% DMSO or 16% PIM were 70.7 ± 25.4 (range, 37.8–115.5) and 67.7 ± 15.7 (range, 48–86), respectively.

To quantify the total number of cells in the colonies, the colonies were harvested and dissociated, and the cells were analyzed by flow cytometry. Antibodies against CD45 and CD71 (transferrin receptor) were used as markers for myeloid colonies (granulocyte, macrophage, and megakaryocytes) and erythroid colonies (red blood cell precursor cells), respectively. As depicted in Fig. 3C, we found no significant differences in the number of CD45+ CD71– ($p = 0.134$), CD45–CD71+ ($p = 0.084$), or CD45–CD71– cells ($p = 0.054$) generated from the samples cryopreserved with either 10% DMSO or 16% PIM.

DISCUSSION

In this study, we demonstrated that PIM, which is a low-molecular-weight carbohydrate already in clinical use, exhibited equivalent efficiency to DMSO when used for the cryopreservation of HPC(A) samples. We found no differences with respect to CD34 cell recovery, CD34 subpopulation frequencies, or CFU-forming potential between cells cryopreserved in PIM versus DMSO. Thus, the results indicated that PIM could replace DMSO as a useful and safe CPA.

Cryopreservation of HSC products for clinical use is already a well-established procedure used for autologous or allogeneic HSCT of primarily cancer patients. Additionally, a rapidly growing number of potential new cellular therapies involve cryopreservation of cell products before clinical use. So far, DMSO has been acknowledged as the "gold standard" of CPAs. However, DMSO-related adverse reactions and cell toxicity has led to an increasing focus on the development of new and nontoxic CPAs.

While investigating PIM as an alternative to DMSO, we found a mean postthaw recovery of viable CD34+ cells of approximately 60% for both DMSO and PIM. This finding was in accordance with other reports on CD34+ cells cryopreserved with DMSO demonstrating mean values of postthaw recovery of 52% to 87%.[19-22] Interestingly, although the mean recovery values of DMSO and PIM were equivalent, we found a considerably wider range in postthaw recovery for DMSO (11%-97%) than for PIM (46%-77%). This result was similar to those of some other studies on DMSO that demonstrated a postthaw recovery of 12% to 252%[23] and 20% to 100%.[12] Some variation between reports may reflect whether samples have been frozen by use of an electronically controlled-rate freezer or a freezing box or whether the cell product was cryopreserved in freezing bags, in cryovials where the sample is taken from a freezing bag or as samples directly cryopreserved in cryovials as in this study. We experienced that the latter may show somewhat lower postthaw recovery.

Recommendations regarding the number of CD34+ cells to be infused to ensure engraftment of almost all HPC(A) grafts are based on the empirical prefreeze numbers of viable CD34+ cells, with the most commonly used limit set at 2×10^6 CD34+ cells/kg. Thus, taking into account the wide range of postthaw recovery for cells cryopreserved in DMSO, this number could reflect a general overestimation of the cells needed for most patients. Thus, using a CPA with lower variability, such as PIM, could lead to a reduction in the recommended lower limit of 2×10^6 CD34+ cells/kg, which in turn could spare some patients the inconvenience and potential complications of an extra day of stimulation and collection and save costs associated with treatment and hospitalization.

The surface marker CD34, although correlating well with HSC engraftment of HPC(A) products, represents a rather heterogeneous population consisting of various types of committed HPCs and rare population of HSCs.[24] Therefore, it is relevant to compare the recovery of different CD34+ subpopulations when testing new CPAs. In this study, we found similar CD34+ subpopulations representing functional HSCs and MPPs from leukapheresis products cryopreserved with either PIM or DMSO, which is why both CPAs are expected to maintain comparable

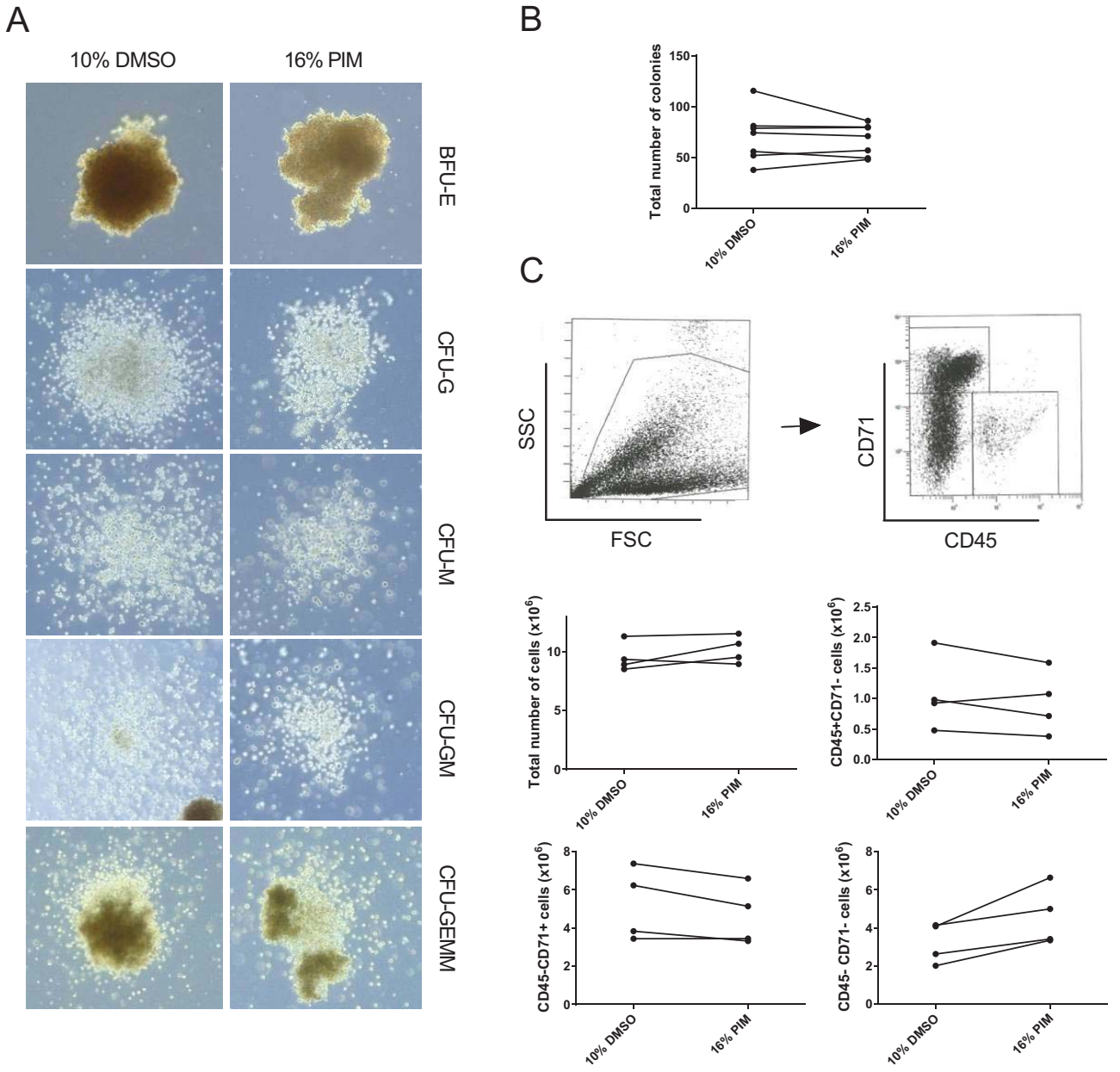

Fig. 3. Colony-forming potential of cryopreserved progenitor cells in apheresis samples. (A) The pictures show the different types of colonies generated from apheresis samples cryopreserved with DMSO or PIM. (B) The total number of colonies generated from cryopreserved samples (n = 7). (C) The total number of cells in all of the colonies in each well and the cells positive or negative for the surface markers CD45 and CD71 (n = 4). We found no significant differences in the number of CD45+CD71– ($p = 0.134$), CD45–CD71+ ($p = 0.084$), or CD45–CD71– cells ($p = 0.054$) generated from the samples cryopreserved with either 10% DMSO or 16% PIM.

number of functional HSCs and long-term engraftment potential.

This assumption is supported by results showing that the viable HPCs after cryopreservation with DMSO or PIM contain comparable number of CFCs. To estimate the total number of cells in the colonies and the cells in the red and white colonies individually, we adopted a new approach using flow cytometry analysis of all colonies in the well. The CD45 + CD71– gate mainly contained macrophages, granulocytes, and megakaryocytes and was clearly defined. Defining the CD71highCD45– and CD71low/–CD45– gates was more difficult, because cells undergoing erythroid maturation gradually lose their CD71 expression concomitant with the increased expression of CD235a

(glycophorin A). We used this phenotype to confirm the development of cells into the erythroid lineage (data not shown). Altogether, we found no significant differences between HPCs cryopreserved in DMSO or PIM with respect to colony numbers, the total number of cells within the colonies and the distribution of cells differentiating into the myeloid and erythroid lineages. DMSO works well as a CPA for most cell types, but can lead to undesirable adverse reactions and DMSO-induced cell toxicity as mentioned above.

PIM is biocompatible and well-tolerated in humans[25,26] and is approved for the prevention of immunologic side effects from subsequent infusion of high-molecular-weight dextran formulations.[27] Additionally, the use of PIM for the cryopreservation of HPC(A) products may eliminate the need to infuse ice-cold cells that is required for DMSO-containing products, because no cell toxicity has been linked to PIM. Finally, the low-molecular-weight PIM is rapidly and completely excreted by glomerular filtration,[28] and in contrast to DMSO, PIM does not generate potentially harmful metabolites when being degraded in the human body.[28]

The mechanism by which PIM may act as a successful CPA of CD34+ cells has not yet been investigated. PIM has a mean weight of 1080 Da (5–6 glucose units), but the weight distribution is wider, ranging from single glucose molecules to polysaccharide chains of more than 15 units of glucose. Thus, we assume that PIM remains in the extracellular space and participates in cell dehydration, thereby preventing intracellular ice crystal formation and stabilizing the cell membrane. In support of an extracellular presence of PIM, we found both a lower osmolarity of PIM cryomedium compared to DMSO cryomedium and smaller cells following exposure to PIM compared to DMSO (data not shown), both before freezing and after thawing.

The cryoprotective effect could be the result of several synergistic properties (e.g., the ability of disaccharides to interact with the polar head groups of phospholipids and stabilize membranes during hypertonic exposure as ice crystals grow[29,30] and larger CPAs that increase viscosity and reduce osmotic stress[31]). The contribution of active transport mechanisms such as GLUT-facilitated glucose uptake or pinocytosis is expected to be negligible when cells and solutions are kept on ice during the freezing procedure. All together, these mechanisms seem to be sufficient to protect most cells from cryoinjury.

Using a CPA unable to permeate the cell membrane could prove an advantage, as it has repeatedly been shown that DMSO affects the expression of stem cell specific genes, induces differentiation, and causes changes of the epigenetic landscape.[10-13] This is most likely an effect caused by the ability of DMSO to enter the cell, emphasized by the ability of DMSO to increase the activity of DNA methyltransferase 3a.[14] It is reasonable to hypothesize that these disadvantageous effects on the cells will be less pronounced using a nonpermeating CPA that is unable to directly interact with the intracellular machinery.

In conclusion, this study demonstrates that HPCs cryopreserved in PIM have a postthaw survival and in vitro function comparable to DMSO, indicating that PIM potentially could replace DMSO as a safer cryoprotectant. However, more preclinical experiments, such as the study of engraftment kinetics in a mouse model, are needed to support our findings before PIM can be used in the clinic.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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