Short Report

Reduced dimethyl sulfoxide concentrations successfully cryopreserve human hematopoietic stem cells with multi-lineage long-term engraftment ability in mice

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ABSTRACT

Background aims: The cryopreservation of hematopoietic stem cells (HSCs) in dimethyl sulfoxide (DMSO) is used widely, but DMSO toxicity in transplant patients and the effects of DMSO on the normal function of cryopreserved cells are concerns. To address these issues, *in vitro* and clinical studies have explored using reduced concentrations of DMSO for cryopreservation. However, the effect of reducing DMSO concentration on the efficient cryopreservation of HSCs has not been directly measured.

Methods: Cryopreservation of human bone marrow using 10%, 7.5% and 5% DMSO concentrations was examined. Cell counting, flow cytometry and colony assays were used to analyze different cell populations. The recovery of stem cells was enumerated using extreme limiting dilution analysis of long-term multi-lineage engraftment in immunodeficient mice. Four different methods of analyzing human engraftment were compared to ascertain stem cell engraftment: (i) engraftment of CD33⁺ myeloid, CD19⁺ B-lymphoid, CD235a⁺ erythroid and CD34⁺ progenitors; (ii) engraftment of the same four populations plus CD41⁺CD42b⁺ platelets; (iii) engraftment of CD34⁺CD133⁺ cells; and (iv) engraftment of CD34⁺CD38[−] cells.

Results: Hematopoietic colony-forming, CD34⁺CD133⁺, CD34⁺CD133⁺ and CD34⁺CD38[−] cells were as well preserved with 5% DMSO as they were with the higher concentrations tested. The estimates of stem cell frequencies made in the xenogeneic transplant model did not show any significant detrimental effect of using lower concentrations of DMSO. Comparison of the different methods of gauging stem cell engraftment in mice led to different estimates of stem cell numbers, but overall, all measures found that reduced concentrations of DMSO supported the cryopreservation of HSCs.

Conclusion: Cryopreservation of HSCs in DMSO concentrations as low as 5% is effective.

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Introduction

Hematopoietic stem cell (HSC) transplantation is a widely used treatment for patients suffering from inherited diseases, cancer and other acquired diseases [1]. Cryopreservation with media containing dimethyl sulfoxide (DMSO) is a critical part of the transplantation procedure, as the use of freshly harvested cells is often not possible. DMSO is a membrane-permeable cryopreservation agent that functions by reducing the crystallization of water that otherwise results in the concentration of solutes and mechanical damage leading to cell death [2]. Although an effective cryopreservation agent, DMSO can also release contaminants from plasticware [3] and affect the

normal functioning of cells [4,5], and DMSO-containing transplants can cause a multitude of patient side effects [6–10]. These adverse effects can include allergic reactions, owing to histamine release; gastrointestinal reactions, such as nausea, vomiting, diarrhea, pain and cramping; cardiovascular and respiratory reactions, such as hypotension, hypertension, decreased heart rate, bradycardia and respiratory symptoms; and other adverse reactions, including hyponatremia, urethral irritation, headache, fever, chills, weakness, sedation and even death.

Reducing the concentration of DMSO used for cryopreservation is a straightforward means to mitigate its detrimental effects on cells and patients. Although cryopreservation in freezing medium with 10% DMSO may be considered the historical standard [11–16], recent research has suggested that lower levels may be used effectively. Reducing DMSO levels to 5% has been shown to be effective in maintaining erythroid precursors from umbilical cord blood [17].

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Similarly, erythroid and myeloid colony-forming cells (CFCs) have been shown to be maintained in cryopreservation media with only 5–7.5% DMSO [18–21]. Flow cytometric analyses of CD34⁺ precursors have further demonstrated enhanced viability when cells are frozen in 5% rather than 10% DMSO [22].

Clinical results from grafts cryopreserved with reduced DMSO concentrations have been positive. In cancer patients receiving autologous transplants, the time to engraftment is similar for grafts cryopreserved in 5% or 10% DMSO, and long-term recoveries are also similar [23,24]. Three concentrations of DMSO (10%, 7.5% and 5%) were tested in a prospective randomized study of cancer patients, and all concentrations were shown to be equally effective in preserving the cells responsible for timely peripheral blood cell reconstitution [25]. Moreover, adverse events associated with stem cell infusion were significantly reduced from 41.7% with 10% DMSO to 19.1% with 5% DMSO. Nonetheless, a diversity of practices for the cryopreservation of HSCs still exist, with DMSO most often used at a concentration of 5–10% [26].

In determining the effects of cryopreservation media on HSCs, the primary difficulty is the lack of definitive *in vitro* measures of these cells. Flow cytometry and CFC assays provide information on progenitors—an important graft component—but these are surrogate assays of the effects of cryopreservation on HSCs. Clinical measures such as time to engraftment of leukocytes and platelets and even hematopoietic engraftment after several years are insensitive measures of the robustness of the HSC pool, as they are only sensitive when hematopoietic engraftment and recovery are poor, which transplant protocols actively aim to avoid [27]. In light of these limitations, the authors have used a xenogeneic transplant model to measure the effects of DMSO concentration on the cryopreservation of human HSCs. Using immunodeficient mice, the numbers of functional HSCs recovered from cryopreservation with 5%, 7.5% and 10% DMSO were quantified using extreme limiting dilution analysis (ELDA) [28].

Methods

Human bone marrow

Mid-gestation bone marrow was used in this study owing to the sufficient quantity of cells that could be recovered from a single specimen and the ability of these cells to vigorously engraft immunodeficient mice [29]. Samples came from San Francisco General Hospital under approval of the institutional review board at the University of California San Francisco (institutional review board no. 10-00768) in accordance with the Declaration of Helsinki. Written informed consent was obtained by clinic staff, but donors remained anonymous to the authors of this study. The gestational age of the specimens (17–22 weeks) was estimated based on foot length measurements.

Cryopreservation and cell recovery

Cryopreservation was performed using a new ampule of DMSO (D2650; Sigma-Aldrich, St Louis, MO, USA) diluted in heat-inactivated fetal bovine serum at 5%, 7.5% or 10% final concentration. CryoStor CS10 (BioLife Solutions, Bothell, WA, USA), a commercial current Good Manufacturing Practice-grade cryopreservative with 10% DMSO and defined components, was also used. Cells were frozen at 9×10^6 cells/mL to 19×10^6 cells/mL, centrifuged, depleted of media and suspended in 1 mL of cryoprotectant. Within 5 min, cryovials were placed into CoolCell FTS30 freezing containers (Corning, Corning, NY, USA), which provide a $-1^\circ\text{C}/\text{min}$ freezing rate, and transferred to a -80°C freezer. One day later, the cells were moved to liquid nitrogen for 22–56 days.

Cells were warmed quickly in a 37°C water bath until almost fully thawed and then slowly mixed with warm phosphate-buffered saline. Cells were washed free of DMSO by $200 \times g$ centrifugation at 4°C for 5 min to avoid the effects of DMSO in culture or transplanted mice.

Cell counting and flow cytometric analysis

A Cellometer K2 (Nexcelom Bioscience, Lawrence, MA, USA) was used to count cells, with viability measured based on Acridine Orange/Propidium Iodide staining. Flow cytometry was performed as described [30]. Antibodies used were purchased from BioLegend (San Diego, CA, USA) and Miltenyi Biotec (Auburn, CA, USA) as listed in supplementary Table 1. Flow cytometry data were analyzed using FlowJo v9 software (FlowJo, Ashland, OR, USA).

Cell cultures

Live cell numbers and viability frequencies were determined after thawing and then culturing in 24-well plates at 0.5×10^6 cells to 1.0×10^6 cells in 1 mL using serum-free medium [31] with recombinant human IL-3 (20 ng/mL) and kit ligand (50 ng/mL) with both cytokines purchased from PeproTech (Cranbury, NJ, USA). After 5 days, cells were counted and analyzed by flow cytometry. CFC assays were performed in quadruplicate using the same medium and cytokines suspended in 0.36% low melting temperature SeaPlaque agarose (Lonza, Rockland, ME, USA) and grown in 35 mm² culture dishes (BD Biosciences, Franklin Lakes, NJ, USA). A total of 5.0×10^4 cells were plated for each 1 mL of culture. CFCs were scored after 14 days of growth as progenitors producing at least 50 cells [32].

Xenografting of mice

Animal experimentation had approval and oversight of the institutional animal care and use committee at Labcorp Early Development Laboratories Inc. (San Carlos, CA, USA; animal welfare assurance no. A3367-01) under protocol IAC 2235. Experiments adhered to the principles of the current version of the National Research Council's Guide for the Care and Use of Laboratory Animals. Husbandry conditions have been previously detailed [33]. Euthanasia was performed according to the recommendations of the American Veterinary Medical Association.

Founder NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at Vitalant Research Institute. At least 3 days prior to irradiation, the standard rodent diet was exchanged for irradiated Global 2018 rodent diet with 4100 ppm Uniprim (Envigo, Indianapolis, IN, USA) for 1 month. NSG mice ≥ 8 weeks old were irradiated with 175 cGy x-rays and approximately 3 h later transplanted with 1×10^6 cells, 3×10^5 cells, 1×10^5 cells or 3×10^4 cells as described [30]. A single bone marrow specimen was used for each of the two experiments. In the first experiment, mice of both sexes were distributed among the groups, and male mice were used in the second experiment.

Engraftment was analyzed ≥ 12 weeks after transplant by flow cytometry as previously detailed [30]. Human leukocytes were identified by CD45 or $\beta 2$ -microglobulin expression and lack of strong mouse marker (TER-119, CD45 and H-2Kd) expression. Engraftment was defined as the presence of CD33⁺ myeloid, CD235⁺ erythroid, CD19⁺ B-lymphoid and CD34⁺ hematopoietic precursors in the bone marrow. An untransplanted mouse was analyzed as a negative control. A transplanted mouse with more than five positive events for each of the four human antigens was considered to be fully engrafted [34]. The overall frequency of engraftment in the bone marrow was determined based on the frequency of human leukocytes and erythrocytes. Additional human lineages and precursor subsets were analyzed in some transplant recipients, including analysis of whole spleen cells, and used to perform ELDA.

Data presentation and statistical analysis

Statistical analysis and charting were performed using Prism 9 (GraphPad Software, San Diego, CA, USA). The *in vitro* effects of different

cryopreservation conditions were compared using one-way analysis of variance with Tukey's multiple comparison test. ELDA was used to quantify HSC frequencies, and the overall tests of differences in stem cell frequencies and pairwise comparisons were made using online software provided by Hu and Smyth [28]. A two-tailed paired *t*-test was used to compare bone marrow and spleen erythrocyte measurements. A $P \leq 0.05$ was considered significant. Data are presented as mean $\pm$ standard error, with individual data shown. Heat maps were created using the mean results for different transplant groups.

Results

In vitro measures of progenitor survival after cryopreservation

With the exception of one measure, the recovery of cryopreserved bone marrow cells and progenitors did not differ significantly among the three concentrations of DMSO tested. After thawing, live cells were counted and colony-forming unit cell assays performed (Figure 1A). Reanimated cells were also placed in liquid culture to allow time for apoptosis to occur in damaged cells and to permit progenitor expansion to multiply minor differences among the groups. Flow cytometry was used to enumerate CD34^{+/+} progenitors as well as two subpopulations of primitive precursors containing HSCs (Figure 1B). With the exception of fewer CD34⁺CD38⁻ cells seen in the 10% DMSO group compared with the 7.5% DMSO group, no differences were observed in the cell fractions recovered from the cultures (Figure 1C).

Effects of DMSO concentration on HSCs

A total of 92 immunodeficient mice were transplanted with four doses of bone marrow cells cryopreserved using three concentrations of DMSO. Engraftment was evaluated 135 days after transplant based on the presence of four cell populations (Figure 2A), and ELDA was performed (Figure 2B). The highest frequency of HSCs was observed in the 5% DMSO group, but the difference was not significant. Similarly, in a second experiment, a total of 93 mice were analyzed 84 days after transplant, with the highest frequency of HSCs observed in the 7.5% DMSO group, but, again, there were no significant differences among the four groups (Figure 2C). CryoStor CS10 was tested as an additional cryopreservation agent in the second experiment and performed very similar to

10% DMSO. Overall, human engraftment levels in the bone marrow for the different groups of mice were similar for different cell doses, with a tendency toward lower percentages of DMSO resulting in the highest levels of chimerism (Figure 2D,E).

Evaluation of alternative methods of gauging HSC engraftment

Among the four cell types used to measure HSC engraftment in NSG mice, erythrocytes were generally the least frequently noted in the bone marrow. The authors examined whether splenocytes would be a better source of mature erythrocytes (Figure 3A). However, pairwise evaluations of the frequencies and numbers of CD235a⁺ events in the bone marrow and spleen clearly indicated that the former was a richer source of human erythrocytes.

The inclusion of CD41⁺CD42b⁺ platelet engraftment in the criteria for determining HSC engraftment was examined (Figure 3B). Fewer mice were deemed engrafted using this five-marker analysis, resulting in estimates of HSC frequencies that were decreased by more than half. In this analysis, the 5% DMSO group had the highest HSC content, whereas the 7.5% DMSO group had more HSCs based on the four-marker analysis (Figure 2C). Nonetheless, no significant differences were observed among the groups by including platelets in the engraftment criteria.

HSC engraftment was also evaluated by the presence of either of two primitive progenitor populations alone. ELDA, based on only CD34⁺CD133⁺ cell engraftment (Figure 3C), estimated the highest frequency of HSCs in the 7.5% DMSO group, which differed significantly from the 5% DMSO ($P = 0.195$) and CryoStor CS10 ($P = 0.0269$) groups but not the 10% DMSO group ($P = 0.0541$). Analysis using the more infrequent CD34⁺CD38⁻ cell population resulted in the lowest estimates of HSC frequencies of all the analyses performed, with the 7.5% DMSO group performing the best (Figure 3D). However, none of the groups differed significantly from one another.

Discussion

Concentrations of DMSO below 10% are being evaluated for the cryopreservation of HSCs in an effort to minimize the unwanted side effects of DMSO on transplant patients. Cells, CD34⁺ progenitors and CFCs from umbilical cord blood and mobilized peripheral blood have been shown to

Figure 1. Recovery of bone marrow cells after cryopreservation. (A) The percentages of live cells recovered after cryopreservation in 5%, 7.5% or 10% DMSO are shown in the left panel. The initial number of cells frozen (100%) is indicated by the vertical dashed line. The number of CFU-C's recovered after thawing per 5.0×10^4 cryopreserved cells is shown in the right panel. (B) Representative flow cytometric analyses of cells obtained from cultures of bone marrow cryopreserved with 7.5% DMSO showing gates used to define total CD34^{+/+} and CD34^{+/+}CD133⁺ cells (left plot) and CD34^{+/+}CD38⁻ cells (right plot). (C) Numbers of live cells and progenitors defined by flow cytometry recovered from cultures of cryopreserved bone marrow. A total of 80 000–500 000 events were collected per sample. Individual data points from four replicate measurements made from a single bone marrow specimen at 17 weeks' gestation are indicated on the charts (A) and (C). ** $P = 0.0089$. CFU-C's, colony-forming unit cells.

Figure 2. Enumeration of cryopreserved HSCs by ELDA. (A) Representative four-marker flow cytometric analysis showing human engraftment in mouse bone marrow. A total of 5000 to >2 000 000 events were collected per sample, depending on the level of human chimerism. (B,C) ELDA for two experiments showing similar HSC frequencies among the groups, as indicated by overlapping confidence intervals (dashed lines). Estimated HSC frequencies are indicated in the legends. (D,E) Heat map representations of mean human engraftment in the bone marrow for each of the transplant groups. CS10, CryoStor CS10.

be cryopreserved with high recovery rates at DMSO concentrations of 5–7.5% or even lower [17–22]. The authors' results using mid-gestation bone marrow were similar, with various measures of cell and progenitor recovery using DMSO concentrations of 5–7.5% as good as those obtained with 10% DMSO. The effect of DMSO concentration on the recovery of cryopreserved HSCs was evaluated using a xenogeneic transplant model. HSCs were defined by their ability to engraft NSG mice for a minimum of 12 weeks, with evidence of multi-lineage engraftment measured by the presence of myeloid, B-lymphoid and erythroid cells. Detection of CD34⁺ progenitors was a fourth engraftment criterion to further demonstrate active hematopoiesis. No significant differences in the frequencies of HSCs, as measured by ELDA [28], were apparent among the DMSO concentrations tested. Indeed, although not significant, the estimated frequencies of HSCs were up to 2.4-fold higher using 5–7.5% DMSO compared with 10% DMSO. The levels of human engraftment also tended to be higher in mice receiving grafts cryopreserved with 5% or 7.5%

DMSO, further indicating that cryopreservation can be effectively performed using these lower DMSO concentrations without loss of HSCs.

The criteria used to assess human HSC engraftment in immunodeficient mice are not standardized, vary by laboratory and have evolved with the development of immunodeficient mouse strains. Some past studies have simply evaluated overall human engraftment (CD45⁺ or myeloid (CD33⁺) and lymphoid (CD19⁺, CD3⁺) engraftment combined [35,36], whereas others have analyzed a wider spectrum of blood cells [37,38]. The authors have previously observed that some engrafted mice may harbor only a single lineage of lymphocytes or monocytic cells; thus, longevity of these cells makes their detection alone insufficient evidence for HSC engraftment [34]. Although erythrocytes have a long life span, their survival in the circulation of NSG mice is short [33,39]. Thus, the authors' observation of fewer of these cells in the spleen than the bone marrow was not surprising. Nonetheless, the detection of erythropoiesis in the bone marrow, especially when combined with the observation of

Figure 3. Additional engraftment parameters and ELDA. (A) Detection of erythrocytes in the spleen and comparison with the frequencies and numbers observed in the bone marrow. At least 500 000 events were collected per sample. Data shown include only positive observations. (B) Detection of platelets in the spleen and ELDA based on five-marker analysis compared with four-marker analysis shown in Figure 2C. ELDA analysis based on detection of only CD34⁺⁺CD133⁺ (C) or CD34⁺⁺CD38⁻ (D) cells. Heat map representations of the mean frequencies of CD34⁺⁺CD133⁺ and CD34⁺⁺CD38⁻ cells in the bone marrow are shown for each of the transplant groups. *****P* < 0.0001. CS10, CryoStor CS10.

CD34⁺ cells, is a reliable indicator of ongoing hematopoiesis and HSC engraftment.

The authors tested other criteria for determining HSC engraftment. Expanding the criteria to include platelets reduced the

frequency estimates of HSCs but did not change the overall findings that showed reduced DMSO percentages to be as effective as 10% DMSO in cryopreservation. Using the observed presence of primitive progenitor compartments (CD34⁺⁺CD133⁺ and CD34⁺⁺CD38⁻ cells)

rather than multi-lineage analysis as evidence of HSC engraftment also did not challenge the conclusions of this study, with the 7.5% DMSO group showing the highest recovery of HSCs. However, the 5% DMSO group still contained numbers of HSCs that were similar to those observed in the two groups with 10% DMSO.

Conclusions

This study provides quantitative evidence that human HSCs are effectively cryopreserved using DMSO concentrations as low as 5%. There are ongoing efforts to further reduce DMSO toxicity by reducing concentrations through the addition of other cryoprotective agents or by washing the thawed cells [5,10]. The design of new cryopreservation agents or methods of freezing may eventually lead to DMSO-free cryopreservation of HSCs [10,40,41]. Until such time, current clinical practice can benefit from reductions in the concentration of DMSO used for HSC cryopreservation.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: RG, MOM and PJN. Acquisition of data: RG, KMA, AH and MOM. Analysis and interpretation of data: RG, KMA, AH, MOM and PJN. Drafting or revising the manuscript: RG, KMA, AH, PJN and MOM. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.07.007.

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