Investigation of Various Methods for the Cryopreservation of Canine Bone Marrow-Derived CD34+ Cells

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ABSTRACT. Optimal condition for the cryopreservation of canine CD34+ cells was explored. Canine bone marrow CD34+ cells were isolated from 5 healthy dogs by a magnetic-activated cell-sorting system using a monoclonal antibody specific to canine CD34. These cells were cryopreserved by 4 different methods: 2 different cryoprotectant solutions—solutions A (fetal bovine serum containing 10% dimethylsulfoxide (DMSO) and B (physiological saline containing 5% DMSO, 6% hydroxyethyl starch, and 4% bovine serum albumin)—were used in combination with 2 different freezing procedures—in a rate-controlled programmed freezer (PF) and in an ordinary freezing container. The cell viability, cell recovery rates, and colony-forming unit (CFU) recovery rates were examined following cryopreservation for 1 week, 4 weeks, and 6 months. The values of these parameters were significantly higher for the CD34+ cells that had been frozen in Solution B than for those that had been frozen in Solution A, regardless of the freezing procedure employed. The highest CFU recovery rate following cryopreservation for 6 months corresponded to the cells that had been cryopreserved with Solution B and frozen in a PF. In conclusion, cryopreservation with Solution B in a PF proved to be the most efficient of the 4 cryopreservation procedures investigated in terms of maintaining the quality of canine bone marrow-derived CD34+ cells. This method will be useful for clinical applications involving the use of canine bone marrow-derived CD34+ cells.

KEY WORDS: bone marrow, canine, CD34+ cells, cryopreservation, hydroxyethyl starch.

Hematopoietic cell transplantation can improve leucopenia and thrombocytopenia following dose-intensified chemotherapy or radiation therapy in human patients with malignancies. This procedure has been reported to produce excellent results for the treatment of non-Hodgkin’s lymphoma in humans [13, 18, 30, 31]. Lymphomas account for more than 80% of all hematologic malignancies in dogs, and the current preferred treatment modality is cyclophosphamide, doxorubicin, vincristine, plus prednisolone (CHOP)-based chemotherapy [12, 40]. Attempts to improve the 1-year median survival rate of this treatment system via modifications in the multidrug protocols have not been successful. Based on the evidence obtained from human medical research, transplantation is currently expected to be the most promising tool for improving the therapeutic outcomes of canine lymphoma [1, 2, 8, 10, 41, 42]. Identification of phenotypes specific to hematopoietic stem cells and progenitors has enabled the separation of primitive populations of these cells, leading to new developments in the field of transplantation medicine. Various stem cell markers such as CD34, Sca-1, c-Kit, and Flk2/Flt3 have been used either singularly or in combination for the sorting of human and murine hematopoietic stem cells [9, 16, 21, 23, 25, 29, 33]. In dogs, CD34 has been identified as a phenotypical marker of canine hematopoietic stem/progenitor cells. The cells can be purified by using antibodies specific to CD34 [26, 27]. The selection of these hematopoietic stem cells also enabled studies of gene therapy; which are transplantations using these cells transduced with certain genes prior to transplantation. Dogs have been used as animal model systems for such research applications [14, 15, 19, 22, 28, 39]. Further, human clinical trials on specific genetic diseases have been also carried out using the hematopoietic stem cells as a target of gene therapy [4, 17].

One of the prerequisites for successful transplantation is the establishment of an efficient cryopreservation system, because transplantations are not always carried out shortly after the cells have been harvested. The conventional cell-freezing procedure involving the use of 10% dimethylsulfoxide (DMSO) solution as a cryoprotectant has been widely accepted as the “gold standard” method. However, this method of cryopreservation poses certain limitations; for example, the cells may aggregate to form clumps during the thawing process, leading to the inevitable loss of viable cells. Further, in vivo toxicity associated with this method has been reported in approximately 20% patients undergoing transplantation [7, 37].

Hydroxyethyl starch (HES) was introduced as a cryoprotectant to overcome the limitations of 10% DMSO solution. Cryoprotectants can be broadly classified into 2 types—“intracellular” cryoprotectants, e.g., DMSO, and “extracellular” cryoprotectants, e.g., HES. Physiological saline solution containing 5% DMSO, 6% HES, and 4% albumin has been used as a cryoprotectant solution for the preservation of human bone marrow and peripheral blood mononuclear cells [24, 37]. The use of this cryoprotectant solution pro-
duced results that were superior to those obtained by using the traditional method with 10% DMSO solution. Further, it produced higher rates of cell recovery after thawing and reduced the side effects associated with the use of these preserved cells. In addition, the use of DMSO-HES solution as a cryoprotectant eliminated the need for a programmed freezer (PF) and liquid nitrogen for the cryopreservation of human cells [24, 37]. In addition to in vitro studies, in vivo studies have been performed to demonstrate that the quality of human bone marrow and peripheral blood cells cryopreserved with DMSO-HES solution is satisfactory [24, 36, 38].

Since many years, 10% DMSO solution has been used as a standard cryoprotectant for bone marrow transplantations in dogs; however, very few studies have evaluated techniques for the cryopreservation of canine cells [3]. To our knowledge, the HES-DMSO solution has never been used to preserve canine cells. In this study, efficacies were compared between 10% and 5% DMSO solutions containing 6% HES and 4% albumin as cryoprotectants for the preservation of purified canine CD34+ cells. Simultaneously, results of freezing these cells in a PF and in an ordinary freezing container were compared as well. Following the cryopreservation of the canine CD34+ cells for up to 6 months, the thawed cells were examined to determine their viability, recovery rate, and colony-forming unit (CFU) recovery rate.

MATERIALS AND METHODS

Animals: This study involved 5 clinically healthy adult beagle dogs aged 1–5 years (2 male and 3 female). The dogs were administered general anesthesia, and bone marrow was aspirated from the humerus and/or femur. This study was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (approval number 181850007).

Purification of CD34+ cells from bone marrow cells: Bone marrow mononuclear cells (BMMCs) were separated by subjecting the aspirated bone marrow cells to density-gradient centrifugation, using Ficoll-Hypaque Plus (GE Healthcare Biosciences Corp., Piscataway, NJ) according to the manufacturer’s protocol. CD34+ cells were separated from the BMMCs by using a magnetic-activated cell-sorting system (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions with some modifications. In brief, the BMMCs were exposed to 20% heat-inactivated canine serum at 4°C for 30 min and were subsequently incubated with 0.05% (w/v) mouse anti-canine CD34 monoclonal antibody (BD Biosciences, San Diego, CA) at 4°C for 15 min. The cells were washed twice and incubated with microbeads coated with 20% (w/v) rat anti-mouse IgG1 (Miltenyi Biotec) at 4°C for 10 min. They were separated by using magnetic bead columns (LS Separation Columns, Miltenyi Biotec) according to the manufacturer’s protocol. The purity of CD34+ cell fractions were analyzed using flow-cytometry (FACS Calibur, BD Biosciences, San Jose, CA). The cells were reacted with biotin-conjugated mouse anti-canine CD34 monoclonal antibody (clone 2E9) (BD Biosciences) followed by streptavidin-PerCP-Cy 5.5 conjugate (BD Biosciences). Biotin-conjugated mouse IgG1 (BD Biosciences) was used as an isotype control. The data were analyzed using a software (CellQuestTM, BD Biosciences).

Freezing and thawing: The CD34+ cells were divided into 4 groups based on the cryoprotectant used (Solution A or B) and the freezing technique employed (with or without a PF). Solution A was fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) containing 10% DMSO (Sigma-Aldrich, St. Louis, MO), and Solution B was physiological saline solution containing 5% DMSO, 6% HES, and 4% bovine serum albumin (BSA, Sigma-Aldrich).

To obtain the Solution A-treated cell groups, the isolated CD34+ cells were suspended in Solution A at a concentration of 1 × 10^7 cells/ml, and the suspension was then dispensed into ten 1.8-ml serum tubes (Sumitomo Bakelite Co., Tokyo, Japan). To obtain the Solution B-treated cell groups, the suspension of isolated CD34+ cells (2 × 10^7 cells/ml in RPMI 1640 medium (Sigma-Aldrich) was mixed with an equal volume of CP-1 solution (physiological saline containing 10% DMSO and 12% HES, Kyokuto Pharmaceutical Industrial, Tokyo, Japan) supplemented with BSA to the concentration of 8%, resulting in the final concentration of 1 × 10^7 cells/ml in a saline solution containing 5% DMSO, 6% HES, and 4% BSA. This cell suspension was dispensed into ten 1.8-ml serum tubes (Sumitomo Bakelite Co.). Five tubes from each solution group were transferred into a freezing container (Nalgene® Cryo 1.8 l) and stored overnight in a freezer at –80°C. Following this, the tubes containing cells frozen in Solution A were transferred into liquid nitrogen at –196°C, while those containing cells frozen in Solution B were maintained in the freezer at –80°C. Next, the remaining 5 tubes for both cell suspensions were frozen in a PF (KRYO 360–1.7; Planer PLC, Middlesex, UK) that had been programmed to cool at a rate of 1°C per min to a temperature of –40°C and then at a rate of 10°C per min to a temperature of –100°C. After the PF had attained the temperature of –100°C, all the tubes were transferred into liquid nitrogen at –196°C.

Following cryopreservation for 1 week, 4 weeks, and 6 months, the cells were thawed for analysis. The tubes were removed from the freezer or liquid nitrogen and were immediately thawed in a water bath at 37°C. These partially thawed cells were resuspended in prewarmed Iscove’s modified Dulbecco’s medium (IMDM) (Sigma–Aldrich) for complete thawing. Following centrifugation of the suspension at 430 × g for 10 min at 4°C, the supernatant containing the cryoprotectant was discarded, and the cells were resuspended in 1 ml IMDM for further analysis.

Cell viability: Following cryopreservation for various durations, the viability of the CD34+ cells was analyzed by performing the trypan blue dye-exclusion test. The viability was calculated as a percentage of the number of unstained
cells with regard to the total number of cells.

Cell recovery rate: The thawed cells were counted to determine the cell recovery rates following cryopreservation for various durations. The recovery rates were calculated as the total number of cells obtained after thawing divided by the total number of frozen cells.

CFU assay: Prior to the CFU assay, phytohemagglutinin (PHA-P, Sigma-Aldrich)-stimulated lymphocyte-conditioned medium (PHA-LCM) was prepared for culture of the canine bone marrow cells. Canine peripheral blood mononuclear cells (2 x 10^6 cells/ml) were cultured for 1 week in IMDM containing 15% FBS and 10 µg/ml PHA-P. The culture supernatant was collected, centrifuged at 200 x g to remove the cells therein, and stored at –80°C until use.

The thawed cells (2.2 x 10^5 cells) were resuspended in 250 µl PHA-LCM and then transferred into 2.5 ml methylcellulose medium containing a mixture of recombinant human cytokines (50 ng/ml stem cell factor (SCF), 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 20 ng/ml G-CSF, 20 ng/ml interleukin (IL)-3, 20 ng/ml IL-6, and 3 U/ml erythropoietin (EPO) (Methocult GF+ 4435) (Stem Cell Technologies Inc., Vancouver, Canada). The cells were briefly mixed with this medium, plated in 35-mm plates (Sumitomo Bakelite Co.), and incubated at 37°C in a humidified atmosphere of 5% CO₂. Following culture for 2 weeks, the total colonies and CFU-granulocyte/monocyte (CFU-GM) were counted under an inverted microscope. CFU-GM was determined based on their morphologies, which was also confirmed by microscopic observation of the cells following staining with Wright-Giemsa solution. The CFU recovery rate at each thawing point was calculated as (number of colonies formed by the thawed cells/number of colonies formed by these cells prior to cryopreservation).

Statistical analysis: Statistical analyses were carried out by performing the Student t test, using the Microsoft Excel software for Mac (Microsoft Co., Redmond, WA).

RESULTS

The purity of CD34⁺ cells in the MACS-sorted CD34⁺-enriched bone marrow fraction obtained from the 5 dogs was 84.5% ± 6.6%.

Cell viability: The cell viability in the CD34⁺-enriched cell fraction was 100% in all of the samples prior to freezing. Following cryopreservation for up to 6 months, the cell viability was significantly higher in the cells that had been cryopreserved in Solution B than in those that had been cryopreserved in Solution A, regardless of whether PF was used in the freezing procedure (Fig. 1). The viability of the cells that were frozen with Solution A in an ordinary freezer was considerably low, at 16.9 ± 15.8%, 0 ± 0%, and 10.9 ± 17.4% following cryopreservation for 1 week, 4 weeks, and 6 months, respectively. Compared to this, usage of PF yielded significantly (p<0.05) higher viabilities after cryopreservation in Solution A for 4 weeks and 6 months. On the other hand, no significant differences were noted with regard to viability among the cells that were cryopreserved in Solution B, regardless of whether a PF was used. Following cryopreservation for 6 months, viability of these cells was approximately 70%, regardless of whether they were frozen with PF.

Recovery rate of CD34⁺-enriched cells: The cell recovery rates from the CD34⁺ cell fraction that had been frozen with Solution B in an ordinary freezer were 118.7 ± 49.4%, 83.6 ± 21.4%, and 111.5 ± 59.5% following cryopreservation for 1 week, 4 weeks, and 6 months, respectively. The cells that had been frozen with Solution B in a PF also yielded high recovery rates, and these rates did not differ significantly from those yielded by the cells that had been frozen with Solution B in an ordinary freezer. The recovery rates of the CD34⁺ cells that had been frozen in Solution A were significantly lower than those of the cells that had been frozen in Solution B, regardless of whether a PF was used (Fig. 2). These results indicated that cryopreservation in Solution B with or without a PF was more efficient than that in Solution A with respect to the CD34⁺ cell recovery rate.

Recovery rates of CFUs: The colony-forming capacity of the CD34⁺ cell fraction following cryopreservation for various durations was determined by performing methylcellulose-CFU assay, and the CFU recovery rates were measured (Fig. 3). The results clearly revealed that Solution B provides significant advantages as a cryoprotectant, regardless of whether a PF is used in the freezing procedure. The total CFU recovery rates of all the cells that had been cryopreserved with Solution B in a PF were 107.6 ± 51.3%, 93.4 ± 54.4%, and 84.9 ± 69.8% following cryopreservation for 1
week, 4 weeks, and 6 months, respectively. These rates were consistently significantly higher than those obtained for the cells that had been cryopreserved in Solution A, regardless of whether a PF was used. Further, the total CFU recovery rates of the cells that had been frozen in Solution B without using a PF were high, at more than 80%, following cryopreservation for a maximum of 4 weeks; however, these rates reduced following cryopreservation for 6 months.

Following cryopreservation for 6 months, the CFU-GM recovery rate from among the cells that had been frozen with Solution B in a PF was 84.9 ± 72.0%.

These results indicated that the cryopreservation in Solution B combined with the use of PF gave the best result in terms of CFU recovery rates for canine CD34+ cell fraction.

DISCUSSION

Solution B containing DMSO, HES, and BSA gave better results with respect to the viability and recovery rate of CD34+ cells and CFU recovery rate than Solution A in which DMSO alone was used as a conventional cryoprotectant. Since a fixed number of thawed cells were seeded for the CFU assay, it is logical to consider that the cell viability, rather than the recovery rate, is more likely to influence the number of colony formation. In humans, a correlation has been shown to exist between the number of CD34+ cells and the number of CFUs and engrafted cells [5, 6, 11, 20, 34], therefore, CD34+ has been accepted as a hallmark of hematopoietic stem cell [21, 26]. On the other hand, one human study described the likelihood of underestimating the engraftability of the cryopreserved graft when the number of CD34+ cells was used as the sole criterion [34]. The discrepancy between the viability and recovery of CD34+ cells following cryopreservation in this study suggests that it is important to evaluate the CFU recovery rate in order to assess the quality of the cryopreserved cells.

The recovery rates of CD34+ cells after cryopreservation were higher in cells frozen in Solution B than those frozen in Solution A. Interesting differences were observed when compared to the results obtained in the other study comparing the cryopreservation procedures using canine BMMCs by the authors. The recovery rates yielded by the BMMCs that were cryopreserved in both solutions A and B were higher when the cells had been frozen in a PF than when they had been frozen in a conventional freezing container. The low recovery rate obtained for CD34+-enriched cells following cryopreservation in Solution A, despite the use of PF, suggests that Solution A may be inadequate as a cryopreservation solution for canine CD34+ cells.

It should be noted that following cryopreservation for 6 months, the viability of the cells that were frozen in Solution B remained high, regardless of whether a PF was used. However, the CFU recovery rate yielded by the cells that had been frozen in an ordinary freezing container was lower than that yielded by the cells that had been frozen in a PF. These results suggest that the duration of cryopreservation should be limited to 1–6 months for effective maintenance of the colony-forming abilities of CD34+ cells frozen with Solution B without PF. Moreover, the cell viability was not necessarily reflective of the colony-forming capacity of the cells, it is possible that other factors such as intracellular signaling molecules and receptors were preserved well during cryopreservation with Solution B in a PF. There has been a report suggesting that factors other than intact cell membranes may be important for colony formation [32]. This difference could be the result of the different final preservation temperatures between the two groups. With PF in this study, the most commonly used temperature program for the cryopreservation of hematopoietic cells was used, which leads to the cell storage in the liquid nitrogen. On the other hand, the storage temperature suggested by the standard protocol for the use of Solution B with ordinary freezer is –80°C. Because cryopreservation studies involving the use of DMSO, HES, and albumin as cryoprotectants [24, 35–38] have not discussed the use of PF, we cannot compare the combined effects of Solution B and a PF observed in this study with the results of these previous studies.
similar studies using cells frozen by PF with a program that ends at \(-80^\circ C\) and stored at \(-80^\circ C\) and comparing to the results obtained in this study may give answers to possible effect of the actual freezing process.

CD34\(^+\) cell is a promising tool for advanced medicine with wide potentials from transplantation medicine to regenerative medicine. By selecting CD34\(^+\) cells, not only conventional transplantation but technologies such as gene transduction for gene therapy can be combined to the transplantation. Such clinical applications are under study in human medicine [17], and similar but experimental studies in dogs have been carried out as well [15, 19, 22].

Thus far, 10% DMSO solution has generally been used for cryopreservation of canine cells for transplantations; however, better results can be expected if more efficient cryopreservation procedures are employed, as shown in this study. For convenience, we used BSA as a substitute for albumin in this in vitro study; however, the use of canine albumin would be more appropriate if the cryopreserved cells are to be applied for practical transplantation procedures in dogs.

In summary, the results of this study indicated that of the 4 cryopreservation procedures investigated, cryopreservation in Solution B along with the use of PF produced the best results in terms of the CFU recovery rates from the canine CD34\(^+\) cell fraction. This cryopreservation method could provide a safer and more secure system for conducting transplantation in dogs that have undergone intensified chemo- or radiation therapy. In addition, since the use of CD34\(^+\) cells is a promising tool for advanced fields of medical research, such as gene therapy, the cryopreservation method reported here may also have useful applications in these fields in the future.

REFERENCES


