Perfusion method for harvesting bone marrow cells from dogs

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Objective—To compare composition and colony formation of bone marrow mononuclear cells (BMMCs) harvested from dogs by means of a new perfusion method and the conventional aspiration method.

Animals—7 healthy adult Beagles.

Procedures—BMMCs were collected from the humeri and femurs of Beagles via perfusion and aspiration methods. Flow cytometric analysis was performed to quantify the presence of contaminant cells from the peripheral blood and the percentage of CD34+ progenitor cells in the BMMCs. A CFU assay was conducted to determine the number of progenitor cells in the BMMCs.

Results—The perfusion method was safely performed in all 7 dogs. Flow cytometric analysis revealed that the percentages of contaminant CD3+CD4+, CD3+CD8+, and CD21+ lymphocytes in BMMCs obtained via perfusion were significantly lower than percentages obtained via aspiration. The percentage of CD34+ cells obtained via perfusion was significantly higher than that obtained via aspiration. In addition, perfusion yielded a significantly higher CFU count than did aspiration.

Conclusions and Clinical Relevance—The perfusion method used in this study can minimize the contamination of bone marrow samples with peripheral blood and was a more efficient means for collecting canine bone marrow progenitor cells than the conventional aspiration method. Therefore, the perfusion method can be more suitable than aspiration for harvesting bone marrow cells for transplantation in dogs. (*Am J Vet Res* 2011;72:1344–1348)

Bone marrow transplantation for the treatment of lymphoma, leukemia, solid tumors, and some non-malignant diseases is an important therapeutic option in human medicine.[1,2] Although the clinical application of BMT in veterinary medicine is still less common than in humans, several studies[3–7] have been conducted to evaluate BMT for the treatment of dogs with spontaneously developing lymphoma. Engraftment and prolonged survival were observed in several dogs that underwent BMT, yet 12% to 81% of dogs died from transplant complications such as failure to recover BMCs and GVHD.[3,5–7]

Bone marrow cells are usually obtained via bone marrow aspiration. However, BMCs become contaminated with cells from peripheral blood during conventional aspiration.[8] In a study[8] involving cynomolgus monkeys, the percentage of T cells from the peripheral blood in the bone marrow exceeded 20%. Because blood contamination of bone marrow specimens reduc-

ABBREVIATIONS

BMC	Bone marrow cell
BMMC	Bone marrow mononuclear cell
BMT	Bone marrow transplantation
GVHD	Graft-versus-host disease
IQR	Interquartile range
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin

es the proportion of pure bone marrow progenitor cells, it may cause unsuccessful engraftment of the bone marrow. Furthermore, the presence of T cells from peripheral blood may trigger GVHD because these contaminant cells can immunologically attack the host, resulting in the need for immunosuppressive treatment.[9–11]

To reduce the contamination by peripheral blood, a new means of BMC harvesting, the perfusion method, was developed in monkeys for possible use in humans.[8] This novel method can markedly reduce blood contamination of BMCs and the time during which a donor is anesthetized for the collection procedure.[8]

With advancements in supportive care, donor-recipient matching, and transplant preservation,[7,12–14] hematopoietic stem cell transplantation is expected to become increasingly common in small animal practice to improve treatment results for various diseases. The purpose of the study reported here was to compare the perfusion method with the aspiration method in dogs with respect to peripheral blood contamination.

Materials and Methods

Animals—Seven healthy adult Beagles aged 3 to 5 years (body weight, 9.3 to 15.0 kg; 2 males and 5 females) were included in this study. Bone marrow cells were first collected from both humeri of all of the dogs, with the perfusion method used for one humerus and the aspiration method used for the other. After a period of 2 months, BMCs were collected from both femurs of the same dogs in a similar manner.

All dogs received sedatives and analgesics before each procedure (medetomidine [20 μg/kg, IM], butorphanol [0.2 mg/kg, IM], and meloxicam [0.2 mg/kg, SC]). Anesthesia was maintained with isoflurane (approx 1.5% in oxygen) for bone marrow collection. A peripheral blood sample was concurrently collected via a jugular vein by use of a 21-gauge needle for each experiment. After each procedure, dogs received meloxicam (0.1 to 0.2 mg/kg, PO, q 24 h for 3 days). The study was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences of the University of Tokyo.

BMC harvesting via the perfusion method—Bone marrow fluid was collected by means of the perfusion method reported for cynomolgus monkeys,[8] with slight modifications. A 15-gauge bone marrow–harvesting needle[a] was inserted into the humeral or femoral head following routine veterinary practices. Another 15-gauge harvesting needle (**Figure 1**),[b] which was engineered specifically for the perfusion method, was inserted into the bone shaft by use of an electric drill at the distal aspect of humerus or femur. After insertion was completed, the inner needle was removed from the bone head and a 50-mL syringe containing 30 U of dalteparin sodium[c]/mL of bone marrow fluid was connected to the outer sheath of the bone-head needle. The inner needle inserted into bone shaft was removed, and a 50-mL syringe containing 50 mL of saline (0.9% NaCl) solution was connected to the outer sheath of the bone-shaft needle. Saline solution was gently injected via the bone-shaft needle as bone marrow fluid was aspirated via the bone-head needle. This procedure was repeated to obtain 100 mL of bone marrow fluid from each bone. One, 7, and 28 days after each harvesting procedure, dogs underwent a complete physical examination, hematologic analysis, and radiographic examination of the thorax and the treated bone for detection of adverse events.

BMC harvesting via the aspiration method—Bone marrow fluid was collected from 1 humerus or femur into saline solution containing 30 U of heparin/mL at a volume ratio of 2 mL of saline solution to 1 mL of marrow as described elsewhere.[4] A total of 30 mL of bone marrow fluid was collected from each bone.

Preparation of BMMCs and PBMCs—Bone marrow fluid was strained through 100-μm mesh strainers to remove fat and other tissue particles. Erythrocytes in marrow suspensions were lysed by adding hemolytic solution (8.26 g of NH_4Cl, 1 g of $KHCO_3$, and 0.037 g of Na_4EDTA/L of distilled water), and the quantity of BMCs was assessed with a hemacytometer. Bone mar-row mononuclear cells and PBMCs were collected via density gradient centrifugation.[d]

Analysis of expression of cell surface antigens on BMMCs and PBMCs—The expression of cell surface antigens on BMMCs and PBMCs was analyzed by use of monoclonal antibodies[e–g] (**Appendix**). Bone marrow mononuclear cells and PBMCs were incubated with each antibody (CD3-biotin, 10 μg/mL; CD4-fluorescein isothiocyanate, 100 μg/mL; CD8-PE, 10 μg/mL; CD21-PE, 10 μg/mL; CD34-PE, 2 μg/mL) in a plastic tube at 4°C for 60 minutes. Biotin-conjugated mouse anti-canine CD3 monoclonal antibody was added, followed by streptavidin conjugate[h] (20 μg/mL) at 4°C for 30 minutes. Cell quantification was performed by use of a flow cytometer and associated software.[g]

CFU assay—The colony-forming ability of the BMMCs was assayed as described elsewhere.[14] Briefly, phytohemagglutinin[i]-stimulated lymphocyte-conditioned medium was prepared by culturing canine PBMCs (2×10^6 cells/mL) in Iscove modified Dulbecco medium[h] containing 15% fetal bovine serum and 10 μg of phytohemagglutinin/mL for 1 week. The culture supernatant was collected as conditioned medium and stored at –80°C until used. The BMMCs (2.2×10^5 cells) were resuspended in 250 μL of conditioned medium and then transferred into 2.5 mL of methylcellulose medium[j] containing a mixture of recombinant human cytokines (50 ng of stem cell factor/mL, 20 ng of granulocyte-macrophage colony-stimulating factor/mL, 20 ng of granulocyte colony stimulating factor/mL, 20 ng of interleukin-3/mL, 20 ng of interleukin-6/mL,
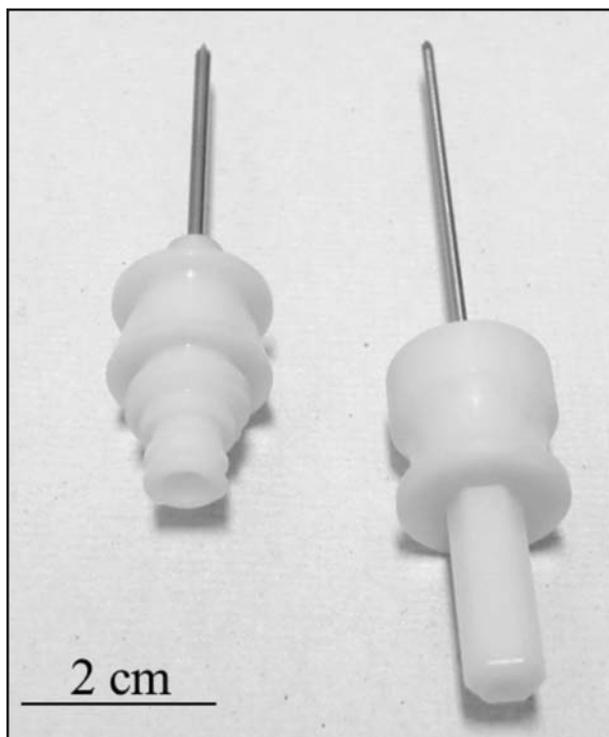

Figure 1—Outer sheath (left) and inner needle (right) of a 15-gauge harvesting needle used for insertion into the shaft of the humerus and femur in dogs that underwent collection of BMCs via a perfusion method. This needle is currently not commercially available.

Table 1—Median (IQR) numbers of T cells (CD3+CD4+, CD3+CD8+, and CD21+) and progenitor cells (CD34+) identified by surface antigens in PBMC and BMMC samples harvested from the humeri (n = 7) and femurs (7) of 7 healthy Beagles by means of a perfusion method and the conventional bone marrow aspiration method.

Cell type	CD3+CD4+	CD3+CD8+	CD21+	CD34+
PBMCs	26.6 (24.4–29.9)	12.1 (10.4–14.6)	14.7 (12.1–16.6)	—
BMMCs via perfusion	3.4 (2.9–5.7)*	3.4 (2.8–5.3)*	7.9 (6.0–12.9)*	5.5 (4.2–7.1)†
BMMCs via aspiration	13.7 (8.2–21.9)	9.2 (6.7–12.2)	12.2 (10.2–14.0)	4.5 (3.1–5.0)

*Value is significantly ($P < 0.02$) lower than the corresponding value for BMMCs harvested via aspiration.
†Value is significantly ($P = 0.006$) higher than the corresponding value for BMMCs harvested via aspiration.
— = Not applicable.

and 3 U of erythropoietin/mL). The cells were vigorously mixed with the methylcellulose medium, plated in 35-mm plates, and incubated at 37°C in a humidified atmosphere of 5% CO_2. After cells had been cultured for 2 weeks, the number of CFUs was counted.

Statistical analysis—Data are reported as median and IQR. The Wilcoxon signed rank test was used to assess statistical differences between the perfusion and aspiration methods with respect to the percentages of CD3+CD4+, CD3+CD8+, CD21+, and CD34+ cells in BMMCs and the CFU count. This test was also used to evaluate differences between humeri and femurs in the number of BMCs harvested. Values of $P < 0.05$ were considered significant.

Results

Animals—Physical examination, hematologic analysis, and radiography performed after the harvesting procedures had been completed revealed that no severe complications such as bone fracture, paralysis, or pulmonary infarction had developed in any of the 7 Beagles. Slight lameness persisting for several minutes was observed in 4 of 7 dogs after recovery from anesthesia (2 after BMC collection from the humerus, 1 after collection from the femur, and 1 after collection from both bone types); however, this lameness was brief, and dogs recovered within 2 hours.

Perfusion method—The entire perfusion procedure was completed within 20 minutes for all dogs. Median (IQR) numbers of BMCs collected by use of the perfusion method from 1 humerus and 1 femur from each dog (7 of each bone in total) were 7.8×10^8 cells (6.8×10^8 cells to 8.1×10^8 cells) and 2.4×10^8 cells (1.7×10^8 cells to 4.0×10^8 cells), respectively. On a dog body weight basis, median values were 6.2×10^7 cells/kg (5.4×10^7 cells/kg to 7.7×10^7 cells/kg) and 2.1×10^7 cells/kg (1.4×10^7 cells/kg to 3.5×10^7 cells/kg), respectively. The number of BMCs harvested from the humerus was significantly ($P = 0.008$) greater than from the femur.

Aspiration method—The entire perfusion procedure was completed within 40 minutes for all dogs and bones. The median number of BMCs collected by use of the aspiration method from 1 humerus and 1 femur (7 of each bone in total) was 3.2×10^8 cells (1.9×10^8 cells to 8.0×10^8 cells) and 1.1×10^8 cells (1.0×10^8 cells to 1.9×10^8 cells), respectively. On a dog body weight basis, median values were 2.4×10^7 cells/kg (1.8×10^7

cells/kg to 6.7×10^7 cells/kg) and 1.1×10^7 cells/kg (1.0×10^7 cells/kg to 1.9×10^7 cells/kg), respectively. The number of BMCs harvested from the humerus was significantly ($P = 0.025$) larger than that from the femur. The total number of BMCs harvested by the aspiration method was significantly ($P = 0.015$) smaller than that harvested by the perfusion method.

Cell surface antigens of BMMCs and PBMCs—The percentages of differentiated lymphocytes (CD3+CD4+, CD3+CD8+, and CD21+ cells) were significantly lower in BMMC samples obtained by the perfusion method than in those obtained by the aspiration method (**Table 1**). The percentage of undifferentiated CD34+ cells was significantly higher in BMMC samples obtained via perfusion than via aspiration. Furthermore, the percentages of T cells (CD3+CD4+ and CD3+CD8+ cells) were > 20% in the BMMC samples collected via aspiration, whereas < 10% of cells in the BMCC samples obtained via perfusion consisted of T cells.

CFU assay—The median CFU count generated from 2.2×10^5 cells was 62.0 CFUs (46.3 to 70.0 CFUs) in BMMC samples obtained via the perfusion method, which was significantly ($P = 0.001$) higher than the count in BMMCs obtained via aspiration (20.5 CFUs [17.3 to 28.0 CFUs]).

Discussion

The primary goal of the present study was to investigate whether a perfusion method for harvesting BMCs from dogs would have advantages over the conventional bone marrow aspiration method. Findings suggested that the perfusion method reduced the intermixing of CD3+CD4+ and CD3+CD8+ T cells with bone marrow fluid, thereby enabling collection of more progenitor cells than could be obtained via aspiration. These results are similar to those of the previous study[8] in cynomolgus monkeys. The perfusion method was conducted safely, and none of the study dogs developed any physical, hematologic, or radiographic signs of clinically important adverse effects.

For successful autologous BMT in dogs, > 5.0×10^7 BMCs/kg are required.[5,7,15–20] In the present study, the perfusion method, which includes the infusion of 100 mL of saline solution (50 mL $\times$ 2) into the bone marrow, enabled the collection of a median of 6.2×10^7 cells/kg from a single humerus, thus meeting this requirement. The time required to collect enough BMCs for BMT via the perfusion method was considerably shorter (< 20 minutes) than via the conventional meth-

od (mean, < 60 minutes[4]). Furthermore, the CFU assay revealed that the BMCs collected via perfusion had a higher progenitor cell count than those collected via aspiration. Thus, a large number of unadulterated BMCs can be harvested with the new method and the number of BMCs required for BMT may be reduced because of the high percentage of hematopoietic progenitor cells obtained.

To the authors' knowledge, the required minimal number of BMCs for allogeneic transplantation in dogs remains uncertain. Recipient dogs were given $> 1.0 \times 10^8$ donor BMCs/kg in previous reports.[6,21,22] Therefore, it is possible that harvesting from ≥ 2 bones would be necessary to ensure a sufficient quantity of BMCs when the perfusion method is used for allogeneic transplantation. Use of granulocyte colony-stimulating factor might increase the yield of the required number of BMCs for BMT, although this was not investigated in the present study.

The quantity of BMCs harvested from the femur was much less than the quantity obtained from the humerus for both methods in our study. This is consistent with findings in monkeys.[23] Additionally, approaching the femur for BMC collection in large or overweight dogs can be challenging. Therefore, the humerus is recommended as an acceptable site for the perfusion method of harvesting.

A needle engineered specifically for the perfusion method was connected to an electric drill to facilitate access to the bone marrow. This needle is not commercially available. Bone marrow biopsy or other needles can be used as substitutes for this technique, but such a substitution will likely make the approach to the bone marrow from the shaft of a long bone more difficult because such instruments are not designed for use with electric drills.

The percentages of CD3+CD4+ and CD3+CD8+ subsets in BMMCs obtained with the aspiration method were nearly 3 to 4 times as high as those obtained with the perfusion method. However, the percentage of CD21+ cells was only 1.5 times as high. The proportions of mature cell subsets obtained with the 2 methods differed, suggesting factors other than peripheral blood contamination of BMC samples may have been involved. One possible explanation is that mature lymphoid cells partially exist in bone marrow, which results in alterations of subset proportions. For example, CD21+ cells might reside in bone marrow more abundantly than mature T cells.

In humans, BMT is used in the treatment of various diseases, ranging from leukemia to autoimmune disorders.[1,2] Associated with a considerable mortality rate, GVHD is a severe complication of BMT. Donor T cells are considered instrumental in the development of GVHD because removal of T cells from donor inocula effectively prevents GVHD.[9–11] Although administration of immunosuppressants can reduce the undesirable consequences of GVHD, such treatments do not completely prevent it. Interestingly, 35% to 45% of humans given human leukocyte antigen–identical marrow grafts from siblings still develop acute GVHD.[24–26] A similar complication of BMT occurs in dogs. Fifty-five percent of dogs given

dog leukocyte antigen–identical marrow grafts from littermates reportedly developed GVHD when they did not receive immunosuppressive treatment after grafting.[24] In another report,[6] 13 of 16 dogs with lymphoma or leukemia that received dog leukocyte antigen–identical allogeneic marrow transplantation after total-body irradiation developed GVHD, even though immunosuppressants were administered. As shown in the present study, the perfusion method may minimize T-cell contamination of BMC samples in dogs. Contamination of BMCs with peripheral blood, as determined by the percentages of CD3+CD4+ and CD3+CD8+ T cells, was significantly lower for the perfusion method (< 10%) than for the aspiration method (> 20%).

Another severe problem associated with BMT is unsuccessful engraftment. Death from subsequent transplant complications can be caused by a failure of BMCs to recover after total-body irradiation. Failure of BMC recovery can occur in 15% to 45% of dogs that undergo BMT.[3] Because the quantities of progenitor cells harvested through the perfusion method were greater than the quantities obtained with the aspiration method in our study, the perfusion method should prevent such complications.

One limitation of the present study was that the subjects were limited to healthy Beagles aged 3 to 5 years. The safety of electrical drill assistance to perform the perfusion method should be evaluated in dogs of various sizes, particularly small dogs, before clinical application is attempted. We performed the perfusion method with only humeri and femurs because BMC are usually obtained from long bones for transplantation in dogs.[3–7,15–22] However, the quantity of collectable BMCs from long bones is expected to decrease with age. In human medicine, BMCs are typically collected from the sternum or iliac crest, and harvesting from in the iliac crest in cynomolgus monkeys can be safely and effectively achieved via perfusion.[23] Additional studies are needed to identify the optimal site from which BMCs can be harvested in dogs.

The perfusion method for harvesting BMCs from dogs was considered technically feasible because it was associated with no severe complications and a shorter period of anesthesia, compared with the aspiration method. Although further development of the perfusion method is required prior to application in clinical trials, we believe that this new technique will contribute to the advancement of the use of BMT in veterinary medicine.

Appendix

Monoclonal antibodies used to detect specific cell types in bone marrow samples.

Antibody clone	Cell specificity-label	Cell phenotype
CA17.2A12[e]	CD3-biotin	T lymphocyte
YKIX302.9[f]	CD4-FITC	T helper
YCATE55.9[f]	CD8-PE	T cytotoxic
CA2.1D[f]	CD21-PE	Mature B lymphocyte
1H6[g]	CD34-PE	Progenitor cell

FITC = Fluorescein isothiocyanate.

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