Use of a centrifugation-based, point-of-care device for production of canine autologous bone marrow and platelet concentrates

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Objectives—To analyze a centrifugation-based, point-of-care device that concentrates canine platelets and bone marrow–derived cells.

Animals—19 adult sexually intact dogs.

Procedures—Anticoagulated peripheral blood (60 mL) and 60 mL of anticoagulated bone marrow aspirate (BMA) were concentrated by centrifugation with the centrifugation-based, point-of-care device to form a platelet and a bone marrow concentrate (BMC) from 11 dogs. Blood samples were analyzed on the basis of hemograms, platelet count, and PCV. The BMA and BMC were analyzed to determine PCV, total nucleated cell count, RBC count, and differential cell counts. The BMC stromal cells were cultured in an osteoinductive medium. Eight additional dogs were used to compare the BMC yield with that in which heparin was infused into the bone marrow before aspiration.

Results—The centrifugation-based, point-of-care device concentrated platelets by 6-fold over baseline (median recovery, 63.1%) with a median of 1,336 X 10⁶ platelets/μL in the 7-mL concentrate. The nucleated cells in BMCs increased 7-fold (median recovery, 42.9%) with a median of 720 X 10⁶ cells/μL in the 4-mL concentrate. The myeloid nucleated cells and mononuclear cells increased significantly in BMCs with a significant decrease in PCV, compared with that of BMAs. Stromal cell cultures expressed an osteoblastic phenotype in culture. Infusion of heparin into the bone marrow eliminated clot formation and created less variation in the yield (median recovery, 61.9%).

Conclusions and Clinical Relevance—Bone marrow–derived cell and platelet-rich concentrates may form bone if delivered in an engineered graft, thus decreasing the need for cancellous bone grafts. (Am J Vet Res 2006;67:1655–1661)

The gold standard for bone grafting in human and companion animal surgery is autologous cancellous bone because of its ability to reliably promote bone formation. Bone grafting can augment healing of large or segmental bone defects, limb-sparing procedures, nonunions, arthrodeses, and acute fractures. Cancellous bone graft collection is associated with complications such as prolonged surgery time, increased infection, pain, seroma formation, hemorrhage, fracture at the donor site, and scarring. Chronic discomfort from the donor site is the most frequently reported complication in humans, with as many as 20% of patients reporting pain 24 months after graft surgery. Other limitations of autologous cancellous grafting are the limited volume and quality in geriatric patients, in pediatric patients, in reharvest procedures from the same surgical site, or when multiple sites must be used for harvest. Therefore, it is desirable to find an alternative procedure to this surgical harvest that would achieve a similar quality of bone formation but with fewer complications.

Fracture healing relies on an adequate population of osteoblastic progenitor cells, the recruitment and osteogenic induction of the progenitor cells at the fracture by local growth factors, neovascularization to deliver and nourish the progenitor cells, a matrix on which these cells can deposit bone, and appropriate mechanical support to the fracture region. The local cell population is a critical component in the inflammatory and repair process. Two easily obtainable autologous cell populations that have potential to stimulate healing when delivered to a surgery site are platelets from peripheral blood and pluripotent cells from bone marrow. Autologous cells are nonimmunologic, pose no risk of disease transfer to the patient, and are readily available. Autologous platelet concentrates are reliably obtained from peripheral blood and platelet-rich concentrates from bone marrow. Platelets harbor growth factors (platelet-derived growth factor, transforming growth factor-β, insulinlike growth factors-I and -II, vascular endothelial cell growth factor, and epidermal growth factor) and, when applied in concentrates, accelerate and promote bone and soft tissue healing.

Bone marrow is a readily available source of progenitor cells that contribute to, or potentially accelerate, bone healing. Current methods such as culture expansion

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACD-A</td>
<td>Citrate dextrose solution A</td>
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<tr>
<td>CBPC</td>
<td>Centrifugation-based, point-of-care</td>
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<tr>
<td>BMA</td>
<td>Bone marrow aspirate</td>
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<td>BMC</td>
<td>Bone marrow concentrate</td>
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and selective cell retention have targeted these cells and used them for formation of bone in a research setting.  

Culturing can expand these cells to a population of millions in a small graft sample. The selective cell retention uses cellular adhesion to a graft matrix to increase connective progenitor cells 3- to 5-fold, compared with the BMA. Neither of these methods currently has a point-of-care availability for the patient, thereby adding additional complexity to an already complex surgery.

The purpose of the study reported here was to analyze the performance of a CBPC device designed to concentrate canine platelets and bone marrow-derived pluripotent cells. For human patients, the prototype of this system efficiently produces an autologous platelet concentrate from peripheral blood samples that has a platelet concentration 4 to 5 times higher than baseline. In this study, we used an updated device. This system is composed of a disposable dual-chamber processor that contains a unique floating shelf. The floating shelf is of a proprietary specific gravity and rises during the initial centrifugation to help separate blood components. Centrifugation is accomplished by use of an automated microprocessor-controlled centrifuge with a swinging bucket design that rapidly separates peripheral blood and bone marrow components. We hypothesized that the CBPC device would concentrate canine platelets to a similar concentration as previously reported in human medicine and concentrate bone marrow cells while maintaining their viability. The platelet and bone marrow–derived pluripotent cell concentrate resulting from low-morbidity procedures (venipuncture and BMA) would be suitable for delivery back to the surgical patient.

Materials and Methods

Animals—Eleven skeletally mature sexually intact dogs (4 males and 7 females) with a mean ± SD age of 13.2 ± 2.0 months and body weight of 23.5 ± 2.9 kg were used. Each dog underwent physical and hematologic examinations, results of which were within reference limits. All procedures were performed according to National Institutes of Health guidelines and with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Anesthesia and preparation—Each dog was premedicated by SC administration of acepromazine (0.02 mg/kg), hydromorphone (0.1 mg/kg), and glycopyrrolate (0.01 mg/kg). Anesthesia was induced by IV administration of propofol and then maintained with isoflurane via an endotracheal tube. Once anesthetized, the dog was placed on its back and both forelimbs were extended caudally and secured. The areas over the proximal portions of the humeri and neck were sterilely draped, and 10 syringes (10 mL) were inserted into the medullary canal of the proximal end of the greater humerus via a small stab incision, and the syringe was filled with bone marrow to a total volume of 7 mL. This procedure was performed 5 times on each humerus starting with the first aspirate at the most proximal end of the greater tubercle and proceeding distally approximately 2 to 4 mm from the previous BMA site. The same person (KMR) performed BMAs on all dogs. All dogs were given a single SC injection of carprofene (4 mg/kg) prior to recovery from general anesthesia.

BMA and BMC—The aseptically prepared areas over the humeri were sterilely draped, and 10 syringes (10 mL) were filled with 0.4 mL of heparin sodium (300 U/mL, diluted with saline [0.9% NaCl] solution). A 15-gauge BMA needle was inserted into the medullary canal of the proximal aspect of the humerus via a small stab incision, and the syringe was filled with bone marrow to a total volume of 7 mL. This procedure was performed 5 times on each humerus starting with the first aspirate at the most proximal end of the greater tubercle and proceeding distally approximately 2 to 4 mm from the previous BMA site. The same person (KMR) performed BMAs on all dogs. All dogs were given a single SC injection of carprofene (4 mg/kg) prior to recovery from general anesthesia.

The remaining plasma was centrifuged for 4 minutes at 1,000 X g, followed by automated decanting and a second centrifugation for 8 minutes at 800 X g. After centrifugation, the processing disposable was removed from the centrifuge and most of the platelet poor plasma (25 to 30 mL) was removed from the plasma chamber with a syringe and needle. The platelet pellet was resuspended in the remaining platelet poor plasma (approx 5 to 7 mL) to produce the platelet concentrate. This concentrate was collected into another syringe, the total volume was recorded, and a sample was collected for platelet cell count and PCV measurement.

Bone marrow–derived stromal cell cultures—The BMC was cultured within 2 hours after collection. Nucleated cells were counted with a hemacytometer, and the cells were assessed for viability by erythrosin B exclusion. Cells were

![Figure 1—Illustration of a processing disposable of the CBPC device after centrifugation and the separation of the blood components as labeled.](image-url)
cultured in 6-well plates at 5 × 10^4 viable nucleated cells/well in α-minimum essential medium containing 10% fetal bovine serum and antimicrobials. On day 3, the medium was exchanged, removing nonattached cells and the medium was supplemented with 10nM dexamethasone to induce an osteogenic phenotype. On day 6, the medium was supplemented with 5mM glycerol 2-phosphate and 17μg/ml ascorbic acid 2-phosphate sesquimagnesium salt. The medium was exchanged every 3 days until confluence. At confluence (11 to 14 days after seeding), cultures were stained to determine whether the BMC contained bone marrow–derived stromal cells that could be induced to have an osteoblastic phenotype. Cultures were stained for alkaline phosphatase activity by use of naphthol AS-MX phosphate and for calcium mineral by use of a von Kossa protocol with silver nitrate. After staining, cultures were scored as positive or negative for cell colonies expressing an osteoblastic phenotype. Four BMC samples had a corresponding negative control culture performed. The medium for the controls did not include dexamethasone, ascorbic acid, or glycerol 2-phosphate.

**Sample analysis**—A hemogram (including total WBC and RBC counts, Hct, and automated differential cell count) and platelet counts were obtained from an automated hematologic analyzer. Packed cell volumes were obtained on blood or bone marrow samples by centrifugation with an electronic impedance counter. Direct anduffy coat smears were prepared from BMA and BMC samples. These were stained with Wright stain and examined by a clinical pathologist (TS). A myeloid-to-erythroid cell ratio and differential cell count (percentage of counted cells) were determined from a 300- to 400-cell count by use of multiple slides and fields (100× oil immersion). For the differential cell count, cells were grouped as differentiating neutrophils (metamyelocytes to segmented neutrophils), proliferative myeloid cells (myeloblasts to myelocytes), differentiating or mature eosinophils, differentiating or mature basophils, lymphocytes, and plasma cells.

**Comparison of aspiration methods**—Two bone marrow aspiration methods were compared to determine whether the yield and consistency of the yield could be improved. For the second aspiration method, an additional 8 hound dogs consisting of 5 females and 3 males were used. Dogs had a mean ± SD age of 19.4 ± 6.6 months and mean ± SD body weight of 28.9 ± 6.1 kg. All methods were as previously described, except for the following differences. Heparin (1.0 mL of 500 U/mL in saline solution) was aspirated into a 12-mL syringe. As soon as the BMA needle was seated in the greater tubercle of the humerus, 0.5 mL of heparin was introduced into the medullary cavity. Five milliliters of bone marrow was aspirated into a 12-mL syringe. The aspirate was then withdrawn though an 18-gauge needle into a 60-mL syringe and placed in the blood chamber of the CBPC device. The aspirate was not filtered. All subsequent methods of cell concentration and counting were as described previously.

**Statistical analysis**—All quantitative measurements were described by the median, minimum, maximum, and quartiles. Platelet count (peripheral blood vs platelet concentrate), nucleated cell count (BMA vs BMC), and differential cell count (BMA vs BMC) were compared with a 1-tailed Wilcoxon signed rank test. Cell yields of BMAs were compared between the 2 aspiration methods with a Wilcoxon rank sum test. Significance was defined at P < 0.05. All analyses were conducted on commercial software.

**Results**

**Animals**—Dogs walked without lameness immediately after recovery from general anesthesia. Dogs had no observed adverse effects from the procedure.

**Platelet concentrate**—The CBPC device provided consistent and easily visible separation of the platelets in all samples and concentrated the platelets in a small volume of plasma. The median final volume of the platelet concentrate was 7.0 mL (range, 5.0 to 8.8 mL). The platelet count significantly (P = 0.004) increased by a median of 596% in the concentrate (median, 1,336 × 10^3 platelets/μL; range, 1,021 to 1,548 × 10^3 platelets/μL) over that in the peripheral blood (median, 224 × 10^3 platelets/μL; range, 168 to 390 × 10^3 platelets/μL; Figure 2). The PCV of the concentrate was a median of 16% (range, 10% to 28%), and the median percent yield of platelets in the concentrate was 63.1% (range, 43.3% to 80.6%).

**BMC**—The system provided consistent and easily visible separation of the bone marrow components in all but 1 sample. The bone marrow was concentrated in a median final volume of 4.0 mL (range, 4.0 to 4.5 mL). Nucleated cells were concentrated in all samples by the centrifugation procedure. A 742% significant (P = 0.004) increase in total nucleated cells was achieved in the BMC (median, 720 × 10^3 cells/μL; range, 130 to 1,036 × 10^3 cells/μL), compared with the BMA (median, 97 × 10^3 cells/μL; range, 49 to 345 × 10^3 cells/μL; Figure 3). The median percent yield of nucleated cells in the BMC, compared with the collected BMA, was 42.9% (range, 6.4% to 82.5%). The BMC of 1 dog did not concentrate (the yield was 6.4%) and 2 other dogs had BMC yields of <20%. By use of this aspiration method, the total median number of nucleated cells in the concentrate that therefore could be delivered in a cell-laden graft was 2.9 × 10^9 with a range of 0.5 to 4.1 × 10^9 cells.

A significant increase in the myeloid-to-erythroid ratio and percentage of proliferative myeloid cells, eosinophils, and lymphocytes was observed in the BMC, compared with the BMA. The PCV significantly decreased in the BMC, compared with the BMA (Table 1).

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**Figure 2**—Box plot of the platelet counts in whole blood samples (Blood) and platelet concentrates (PC) from 11 dogs. The box represents the interquartile range (middle 50% of the data), and the line that bisects the box represents the median. The vertical lines extending from the box represents the range of data values that are within 1.5 times the interquartile range. Asterisk = Extreme values.  

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Bone marrow–derived stromal cell cultures—Cells in the BMC were viable at the time of culturing (median viability, 96%; range, 81% to 100%). All cultures grew to confluence in 14 days, and the cells had a spindle-shape morphology typical of stromal cell cultures. All cultures except the negative controls were positive for alkaline phosphatase activity and mineral content, indicating that the BMC contained osteogenic precursors.

Comparison of aspiration methods—No clots were observed in aspirates collected after heparin was infused into the medullary cavity. The median yield of the BMA collected after heparin was infused into the bone marrow (61.9%; range, 35.9% to 79.7%) was higher than the yield of the original method (42.9%; range, 6.4% to 82.5%), but the difference was not significant. The yield range was narrower for the group that had heparin infusion into the bone marrow prior to aspiration, indicating less variability by use of this technique. All dogs in this group had yields of > 55%, except for a dog with a yield of 35.9% and a second yield of 42.7%. No difference in fold concentrations between the BMA and BMC or total number of cells in the BMC between the 2 aspiration methods was found. By use of the second aspiration method, the total median number of nucleated cells in the concentrate that therefore could be delivered in a cell-laden graft was $2.2 \times 10^9$ with a range of 0.5 to $4.4 \times 10^9$ cells.

Discussion

A CBPC device has not been used before in dogs to concentrate platelets and bone marrow cells. Results of our study indicate that the system is easy to use and autologous platelet concentrations are consistently above the baseline platelet concentrations in the peripheral blood. The 6-fold increase in median platelet concentration in our study is similar to a 4- to 5-fold increase reported in 2 previous studies in which this system was used with human blood samples. The median platelet yield of 63.1% in our study was similar to that reported in an earlier human study (63.4%). However, it is lower than in the more recently reported human study with this system that had a 72% yield. Some of the platelets not in the concentrate are presumably left in the platelet poor plasma in the plasma chamber and with the RBCs in the blood chamber. The system also produced an autologous BMC that had a significant increase in nucleated cells composed of more myeloid and mononuclear cells, while significantly decreasing the amount of RBCs within the sample, compared with that of the baseline aspirates. We speculate that by increasing these cell types, an increase in osteoblastic progenitor cells will occur. In 2 previous studies, mesenchymal stem cells were isolated from a mononuclear cell population obtained from BMAs. To further classify the multipotential characteristics of these cells, cell surface markers for mesenchymal stem cells or induction of cellular differentiation in culture along connective tissue pathways should be performed. We previously reported that this mesenchymal cell population aspirated from canine bone marrow does contain CD34-positive cells.

To optimize the collection of nucleated cells within BMAs, we collected multiple small aspirates from each humerus with the dog in dorsal recumbency. Repositioning of the patient may be required if another surgical position was desired. Alternatively, the collect-

### Table 1—Median differential cell counts (percentage of cells) and the PCV of BMA and BMC from 11 dogs.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample type</th>
<th>Median</th>
<th>Range</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid-to-erythroid cell ratio</td>
<td>BMA</td>
<td>1.3</td>
<td>0.82–2.57</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>BMC</td>
<td>1.9</td>
<td>1.08–2.85</td>
<td>0.039</td>
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<tr>
<td>Proliferative myeloid cells (%)</td>
<td>BMA</td>
<td>4</td>
<td>2–13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMC</td>
<td>7</td>
<td>3–16</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>BMA</td>
<td>3</td>
<td>1–5</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>BMC</td>
<td>4</td>
<td>1–7</td>
<td></td>
</tr>
<tr>
<td>Erythroid precursor cells (%)</td>
<td>BMA</td>
<td>43</td>
<td>28–55</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>BMC</td>
<td>34</td>
<td>26–48</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>BMA</td>
<td>10</td>
<td>4–29</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>BMC</td>
<td>13</td>
<td>9–42</td>
<td></td>
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<tr>
<td>Erythroid precursor cells (%)</td>
<td>BMA</td>
<td>36</td>
<td>30–44</td>
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<tr>
<td></td>
<td>BMC</td>
<td>12</td>
<td>6–26</td>
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All differential cell counts were performed by the same clinical pathologist (TS) to reduce inherent variability in their estimation.

*Only results for which a significant ($P < 0.05$) difference was found between the BMA and BMC are shown. †Results based on 9 dogs.
tion procedure could be performed before the surgery, and the samples could be processed at the time of the surgical preparation and approach. A BMA of ≤ 2 mL from each site is recommended in the human literature, because this will reduce blood contamination and maintain high concentrations of osteoblast progenitor cells. In our study, this BMA volume would require approximately 15 aspirations/humerus, introducing technical difficulties and necessitating the use of almost the whole humerus given the processing volume of the system (60 mL), so we used a larger BMA volume per site. We observed clots in the filter of most BMAs in the first group. We therefore compared this method of aspiration with a method in which a small volume of heparin was infused into bone marrow before aspiration. This method resulted in less variation in the yield and a higher yield of bone marrow cells in the BMC, but the difference was not significant. Also, the total number of cells in the BMC was not significantly different between the 2 methods. It remains to be seen whether other aspiration or concentration methods can result in higher BMC yields that contain significantly increased numbers of bone marrow–derived cells. Fluids were not given IV prior to or during the aspiration procedure to eliminate the effect of hemodilution and bone marrow dilution and to optimize the yield. Fluids could be administered IV after the aspirations and during any surgical procedure that followed.

Favorable features of the CBPC device include the sterile processing disposable, automated 2-step centrifugation, quick sample concentration (14 minutes), minimal and easy to obtain additional equipment, and ease of point-of-care use. Production of concentrates led to no clinical complications, and no morbidity was seen in any of the dogs from the venipuncture or BMA sites. A disadvantage with the system is the large sample size required to use the processing disposable. This was not an issue in the size of dogs in our study, but in small patients, the use would be limited with this size of chamber. A smaller processing disposable is commercially available that requires 20 mL of sample to produce platelet-rich plasma; however, this would yield a smaller volume of concentrate and number of nucleated cells. We did not evaluate the 20-mL disposable, but it likely would be useful for smaller patients.

Bone marrow–derived stromal cell culture results revealed that the CBPC device method maintained cell viability. The BMC cells expanded in culture and differentiated towards an osteoblastic lineage. We used traditional methods to evaluate osteoblastic characteristics of the mesenchymal stem cells that had been cultured in an osteogenic-inducing medium. Quantification of these cells on the basis of DNA, cell counting, colony formation, alkaline phosphatase or osteocalcin concentrations, or mRNA for other phenotypic markers would have provided estimates of how many mesenchymal cells were induced down an osteoblastic lineage but were not undertaken in our study. The aim of our study was simply to demonstrate that the aspirated cells were viable and that they could be induced down an osteoblastic lineage. Additionally, other culture agents like bone morphogenetic protein-2 could be used to induce osteogenic differentiation.

At this time, we do not know how many of these aspirated and concentrated cells would survive in vivo in a fracture site. With a cancellous bone graft, it is estimated that only 10% of the cells transferred to the fracture site will survive. It is unknown exactly how many osteogenic cells or progenitors are required to promote adequate bone healing and what the ideal microenvironment is for these cells. The experimental isolation and culture expansion of pluripotent cells derived from bone marrow that are then combined with an osteoconductive matrix and placed back into the patient have initiated bone formation equivalent to, if not better than, autologous cancellous bone. Many of these cell-based methods require ex vivo culturing, which results in added expense, delay for the graft implantation, and the increased risk of potential contamination. Another, alternative method to cancellous bone grafting is the implantation of selective cell retention grafts. This method uses bone marrow from an aspirate, which is drawn through a synthetic bone matrix to increase the contravasive tissue progenitors within the graft. With this technique, Muschler et al showed that, on average, a 5.6-fold increase in connective tissue progenitors and a 2.3-fold increase in marrow-derived cells occurred. This led to bone healing unions that were comparable to historically reported autologous cancellous bone grafts in dogs. Muschler et al also reported in an earlier study that a 3-fold increase in osteogenic progenitors and 2.5-fold increase in other nucleated cells above naïve concentrations were successful in forming bone when this concentration was combined with a so-called biologic environment at the graft site. The selective cell retention method is quicker and requires less equipment and limited laboratory assistance, compared with the culturing and reimplantation methods. However, Muschler et al reports that equipment modification is needed for practical use in the operating room. Recent evidence suggests that the efficacy of bone healing was positively correlated with the number of progenitor cells in the graft in the treatment of tibial diaphyseal nonunion fractures and avascular necrosis of bone in people with bone marrow–derived cells concentrated in a cell separator. The number of progenitor cells available in BMAs obtained from the iliac crest in these human patients appeared to be less than optimal for healing in the absence of concentration. On the basis of our results, the best yields that could be expected with the CBPC device system are about 80% cell recovery from a BMA after concentration and as high as 90% for platelets. If the bone marrow aspiration method used included infusion of heparin into the bone marrow, the worst yield was 35% for bone marrow–derived cells after concentration. It is difficult to directly compare total numbers of bone marrow cells in the cell concentrate between our methods and those of Hernigou et al because the concentration methods were different and no cell distributions are provided. Roughly speaking, the bone marrow in these young dogs contains 1,000 times as many nucleated cells as human BMAs. It remains to be seen how many nucleated and specifically progenitor cells need to be delivered in an engineered bone graft to a
critical bone defect in dogs to achieve bone healing in a reasonable time frame.

Selective cell retention technology indicates that an autologous BMC could induce bone healing equal to an autologous cancellous bone graft. The veterinary literature contains only a few reports of the usefulness of BMA or platelet concentrates in the clinical setting to stimulate bone production. In human medicine, platelets and BMCs provide noninvasive treatments to stimulate bony healing. During experimental studies, unconcentrated bone marrow added to the surgery site did promote healing but still lagged behind autologous cancellous bone graft.

Ultimately, autologous concentrates such as those produced by the CBPC device may have many clinical uses to stimulate bone healing by minimally invasive methods and reduce the need for cancellous bone graft harvest. Given the viability of the BMCs, stromal cell culture growth, and the increase in nucleated cell counts, we have good reason to believe that this concentrate could induce bone formation in an osteoinductive environment. In preliminary work, we found that when the BMC is combined with tri-calcium phosphate and the platelet concentrate, bone union occurred in 2 of 4 critical (21 mm) experimental fracture gaps in a 4-month period. Further experimental and clinical trials are necessary to completely evaluate the bone healing potential of these concentrates.

References


