OPEN

EXPERIMENTAL

Concentration of Rat Bone Marrow Nucleated Cells Using Hypo-osmotic Hemolysis in Distilled Water

Fumio Ohnishi, MD*
Hiroko Ochiai, MD, PhD†
Kazuo Kishi, MD, PhD‡

Background: Bone marrow aspirates contain primarily red blood cells. To achieve efficient cell transplantation for regeneration, the red blood cells need to be removed from the aspirates. Cell isolation is typically performed using density gradient centrifugation. However, this method entails issues of clinical safety and convenience. This study describes an efficient method to concentrate bone marrow nucleated cells by hypo-osmotic hemolysis.
Methods: The optimal hemolysis conditions were determined by diluting the bone marrow suspensions with distilled water in various dilution ratios. Then, the resulting cell fractions were transplanted in a rat cranial defect model to evaluate their effects on bone formation and their angiogenic effects.
Results: The optimal hemolysis conditions were a 3.3-fold dilution in distilled water and a hypo-osmotic exposure time of 45 seconds. Nucleated cells obtained using this method included granulocytes and mononuclear cells. These cells contain cytoplasmic angiogenic factors, including vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor. In a rat cranial defect model, callus formation and angiogenesis were significantly increased following transplantation of concentrated marrow nucleated cells in this manner.
Conclusions: These results suggest angiogenic and osteogenetic effects of transplanting marrow nucleated cells using this hypo-osmotic method. *(Plast Reconstr Surg Glob Open 2014;2:e184; doi: 10.1097/GOX.0000000000000098; Published online 22 July 2014.)*

Bone marrow aspirates can be used in therapies to treat ischemic tissues, bone fractures, and bony defects. These treatments primarily utilize 2 methods.[1–8] The first involves transplantation of marrow mononuclear cells isolated from aspirates using density gradient centrifugation or a cell separator. The second method involves isolation and culture of bone marrow–derived endothelial progenitor cells or mesenchymal cells. The disadvantages of these methods are the time requirements and safety issues arising from the use of animal-derived materials in culturing or the need for chemicals that are not usually used for clinical applications.

Red blood cells may be removed by erythrocyte sedimentation[9] using a hemagglutination reagent or by hemolysis using ammonium chloride. We speculated that if efficient hypo-osmotic lysis of just the red blood cell fraction could be performed using distilled water, which is readily available and harmless to the human body, then such a method could be easily adapted for

*From the *Department of Plastic and Reconstructive Surgery, Saitama Medical Center, Saitama Medical University, Kawagoe City, Saitama, Japan; †Department of Plastic and Reconstructive Surgery, National Hospital Organization Tokyo Medical Center, Meguro-ward, Tokyo, Japan; and ‡Department of Plastic and Reconstructive Surgery, Keio University School of Medicine, Shinjuku-ward, Tokyo, Japan.*

Received for publication July 31, 2013; accepted March 25, 2014.

DOI: 10.1097/GOX.0000000000000098

Disclosure: *The authors have no financial interest to declare in relation to the content of this article. The Article Processing Charge was paid for by the authors.*

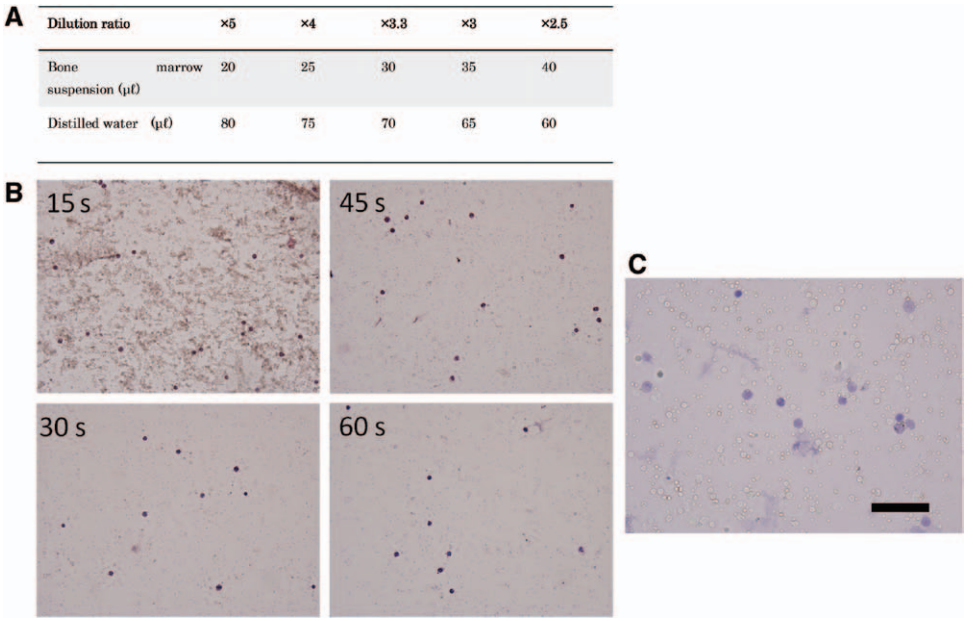

Fig. 1. A, The bone marrow suspensions were diluted with distilled water by various dilution ratios to determine the optimal hemolysis conditions. B, The progress of hemolysis in the 3.3-fold diluted suspension. Many red blood cells remained unhemolyzed after 15 seconds of exposure time to hypo-osmotic conditions. The majority of the red blood cells were lysed after 30–45 seconds, and the number of hemolyzed cells remained almost unchanged after longer time periods. The number of the marrow nucleated cells appeared to be almost constant. The optimal hemolysis conditions were a 3.3-fold dilution in distilled water and a hypo-osmotic exposure time of 45 seconds. C, The cell population obtained using this conditions was stained using trypan blue and consisted of 89.5% ± 1.3% viable cells.

clinical use. In our study, distilled water was added to bone marrow suspensions to keep them in hypo-osmotic conditions for a sufficient period of time to lyse the red blood cells. Subsequently, the suspension was returned to isotonic conditions, and the nucleated cell population was collected. Our studies investigated whether efficient concentration of the bone marrow could be performed using distilled water and also whether transplantation of the resulting cell fractions resulted in angiogenic effects and bone formation.

MATERIALS AND METHODS

Concentration of Marrow Nucleated Cells Using Distilled Water (Distilled Water Method)

Animal care and all experimental procedures were performed in accordance with the Guidelines of Keio University for Animal and Recombinant DNA experiments. The Institutional Animal Care and Use Committee reviewed and approved the experiments. First, appropriate conditions for hemolysis were established by varying the ratio of distilled water to the cell suspension. Retired male breeder F344 rats (CLEA Tokyo, Japan) were used for these experiments. They were anesthetized using intraperitoneal pentobarbital (50 mg/kg). For each rat, an incision was made in the skin just above the iliac crest, and bone marrow was aspirated from the bilateral iliac bones using an 18-gauge needle and a heparinized syringe. The wound after bone marrow aspiration was closed by 4-0 nylon suture. The 0.5 mL of bone marrow obtained from the iliac crest was suspended in phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 5 minutes. The precipitated cell fraction was collected and suspended in PBS.

The bone marrow suspension was diluted with distilled water to achieve a 2.5- to 5.0-fold dilution (total solution volume, 100 μL) under various osmotic pressures (Fig. 1). Ten minutes later, an appropriate amount of 10× PBS was added to the diluted suspension, which was then returned to isotonic conditions to restore osmotic equilibrium. The hemolytic state was evaluated under a stereomicroscope to determine the optimal dilution ratio.

Next, we determined the appropriate length of time for exposure to hypo-osmotic conditions. The hypo-osmotic exposure time was set at 15, 30, 45, or 60 seconds following the addition of distilled water. Giemsa staining and trypan blue staining were carried out for the resulting pellet to determine the fraction of remaining red blood cells and the proportions of viable and nonviable cells. The mononuclear cell fraction was isolated using cell separation

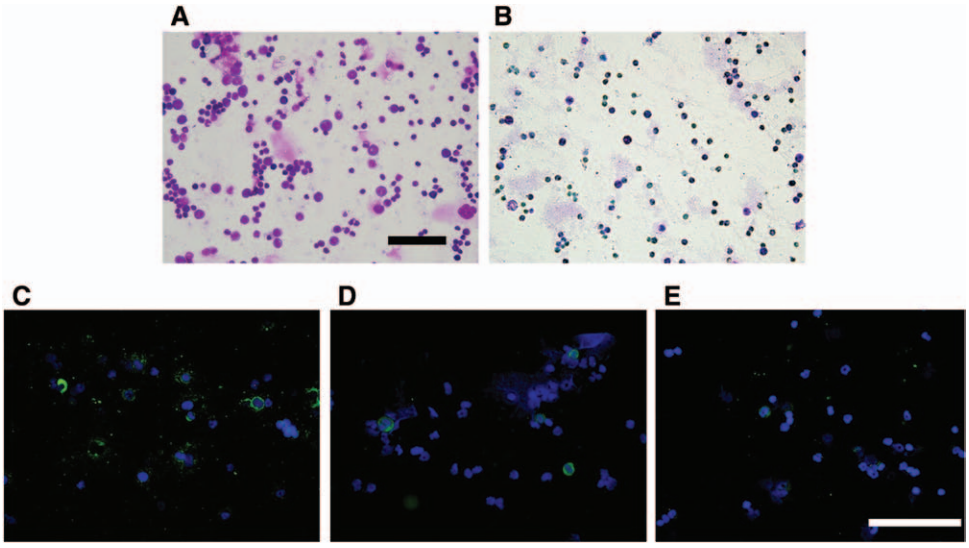

Fig. 2. Comparison of cell populations using Giemsa staining obtained by the concentration method using distilled water (A) and that obtained by density gradient centrifugation (B). Density gradient centrifugation selectively isolated mononuclear cells. The distilled water method yielded not only mononuclear cells but also many cells of the granulocytic series. Immunohistochemical staining was carried out for growth factors from the cell population obtained by the concentration method using distilled water. Immunocytochemical examination indicated the presence of cells with cytoplasmic VEGF (C), bFGF (D), and HGF (E). Bar = 100 μm.

medium (Histopaque 1083; Sigma-Aldrich, Tokyo, Japan). Giemsa staining was carried out on the resulting pellets. Results of treating the pellets using the different methods were then compared.

Immunohistochemistry

Qualitative Properties of Angiogenic Factors from Concentrated Marrow Nucleated Cells Obtained Using the Distilled Water Method

Immunostaining for vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) was performed on samples of concentrated marrow nucleated cells obtained using the distilled water method. Concentrated bone marrow smears were fixed with acetone for 10 minutes and the following primary antibodies were used: goat anti-VEGF antibody (P-20; Santa Cruz, Biotechnology Dallas, TX), goat anti-FGF-2 antibody (N-19; Santa Cruz Biotechnology), and mouse anti-HGF antibody (ab10679; Abcam, Cambridge, UK). The smears were allowed to stand for 50 minutes and then washed with PBS 3 times. Subsequently, biotinylated secondary antibodies were added: anti-goat IgG antibody (Amersham, Piscataway, NJ) for VEGF and bFGF and anti-mouse IgG antibody (VECTASTAIN ABC Kit; Vector, Burlingame, CA) for HGF. The smears were allowed to stand for 30 minutes and then washed with PBS 3 times. Subsequently, they were treated with streptavidin-fluorescein (Amersham). Nuclear staining was performed using Hoechst 33258 solution (Dojindo, Kumamoto, Japan).

Osteogenic Effects of Transplanting Concentrated Marrow Nucleated Cells

An experiment was conducted to evaluate new bone formation using a cranial defect model in retired male breeder F344 rats. The rats were anesthetized using intraperitoneal pentobarbital. For each rat, a midline incision was made over the calvaria and subperiosteal dissection was performed to expose the cranium. A 5 mm in diameter full-thickness defect was created using a round bur in 1 site each on the right and left parietal bones (n = 4). In the right bony defect (hole), the concentrated marrow aspirate was transplanted using fibrin glue as a carrier. In the left bony defect, fibrin glue alone was used to fill the hole as the control defect. Cranial skin was closed immediately after transplantation. Standard x-rays were used to examine the fragments. New bone formation was compared between the treated defects and the control defects using Image J 1.45s (Wayne Rasband, National Institutes of Health, Bethesda, MD) to visualize the x-rays and analyze the areas of calcification 5 and 10 weeks postoperatively. Hematoxylin and eosin staining was performed on tissue sections that included the bony defects and that were collected 5 weeks after surgery. The tissues were examined under a light microscope.

Statistical Analysis

All results are expressed as mean ± SD. Student's *t* test was used for statistical analysis. The significance level was set at $P < 0.05$.

RESULTS

Optimal Hemolysis Conditions

Almost all of the red blood cells were lysed immediately in the 4- and 5-fold diluted bone marrow suspensions in distilled water. In the 2.5-fold diluted suspension, many red blood cells remained unhemolyzed even after 10 minutes. In the 3.3-fold suspension, a majority of the red blood cells had lysed within 1 minute and red blood cell debris was observed. In the 3.3-fold diluted suspension, many red blood cells remained unhemolyzed after 15 seconds of exposure time to hypo-osmotic conditions. The majority of the red blood cells were lysed, however, after 30–45 seconds, and the number of hemolyzed cells remained almost unchanged after longer time periods. The number of the marrow nucleated cells appeared to be almost constant (Fig. 1).

Thus, the optimal hemolysis conditions were a 3.3-fold dilution in distilled water and a hypo-osmotic exposure time of 45 seconds. Pellets obtained using these conditions were stained using trypan blue, and the cell population was 89.5% ± 1.3% viable cells (Fig. 1). The pellets resulting from the distilled water method contained not only mononuclear cells but also many cells of the granulocytic series (Fig. 2), in contrast to the density gradient centrifugation method that yielded a pure mononuclear cell fraction (Fig. 2). Mononuclear cells comprised 67% of the cells in pellets from the distilled water method and 78% of the cells in pellets from the density gradient method. Angiogenic factor immunostaining revealed cells positive for VEGF, bFGF, or HGF in the cytoplasms (Fig. 2).

New Bone Formation in Bony Defects

Macroscopically, callus-like tissues covering the treated and control defects were found 5 weeks postoperatively (Fig. 3). A plain x-ray revealed calcification in the margins of the bony defects (Fig. 3). The extent of calcification was calculated as the calcified area using ImageJ. The calcified areas were larger in the treated defects than in the control defects. The calcified areas of the treated defects were larger than those of the control defects at both 5 and 10 weeks after transplantation (Fig. 3). There was a significant difference in the calcified areas of samples 1–4 between the treated defects and the control defects ($8.7 \pm 1.8\,mm^2$ vs $7.0 \pm 1.2\,mm^2$, respectively; $P < 0.05$) (Fig. 3).

Histologically, the treated defects had fibrous callus-like tissue in some marginal areas of the defects. In contrast, connective tissue was the main constituent in the control defects (Fig. 4). The treated defects had significantly more new blood vessels than the control defects (12.8 ± 3.9 vs 7.5 ± 3.5, respectively; $P < 0.05$) (Fig. 4).

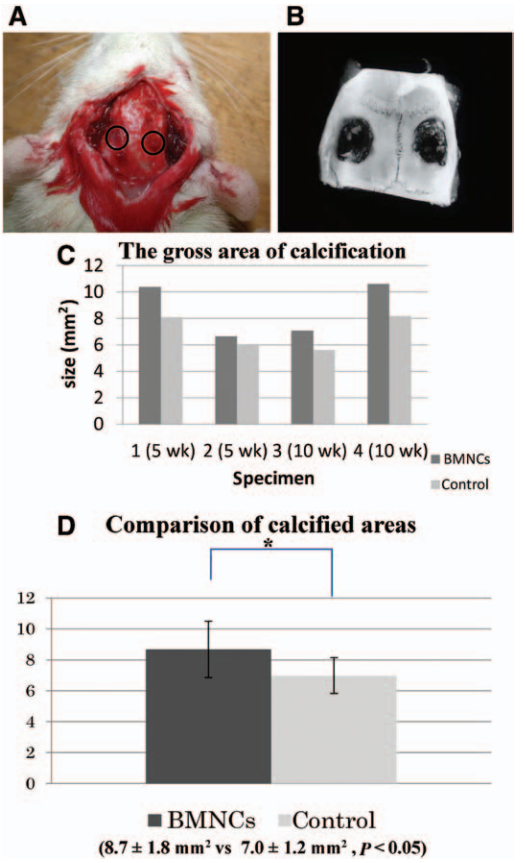

Fig. 3. A, A representative bony defect 5 weeks postoperatively. A thin callus-like tissue was observed in the defect with bone marrow nucleated cell (BMNC) transplantation (right) and in the control defect (left). B, A standard x-ray of a cranial specimen including the bony defect 5 weeks postoperatively. Calcification of callus was observed. C, The area of calcification was calculated using this radiograph and ImageJ. The calcified areas of the treated defects were larger than those of the control defects at both 5 and 10 weeks after transplantation of BMNCs. D, There was a significant difference in the calcified areas of samples 1–4 between the treated defects and the control defects ($8.7 \pm 1.8\,mm^2$ vs $7.0 \pm 1.2\,mm^2$, respectively; $P < 0.05$).

DISCUSSION

The red blood cell membrane is semipermeable. The resistance of this membrane to osmotic pressure differs between red blood cells and nucleated cells. Thus, red blood cells could be lysed under low osmotic pressure without affecting the nucleated cells. Subsequently, the suspensions were returned to isotonic conditions to yield nucleated cells. Only distilled water and PBS were used for this concentration procedure. These materials are easily available in clinical practice and are harmless to the human body.

Under the conditions described herein, approximately 90% of the total nucleated cells were viable. These cells contained angiogenic factors such as VEGF,

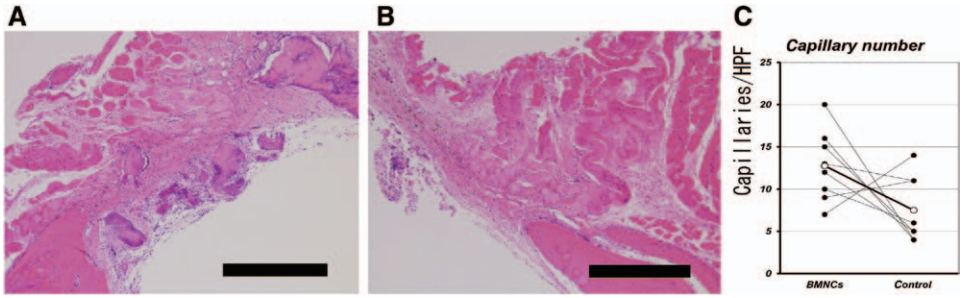

Fig. 4. A, Slightly eosinophilic osteoids and purple calcified areas were observed in the defect treated by bone marrow nucleated cell (BMNC) transplantation. B, Connective tissue was the main constituent in the control defect. Bar = 500 μm. C, The number of blood vessels per field of view was significantly higher in defects treated by BMNC transplantation compared with the control defects (12.8 ± 3.9 vs 7.5 ± 3.5, respectively; $P < 0.05$). HPF, high power field.

bFGF, and HGF, and the number of new vessels increased in the experiment involving new bone formation. These findings suggested angiogenic effects of the nucleated cells concentrated using distilled water.

Clinically, a 30-second hypo-osmotic hemolysis using distilled water[10] and a microfluid protocol[11] have been reported to remove remaining red blood cells from cell fractions obtained by density gradient centrifugation. Damage to nucleated cells is considered to be minimal, but it will be necessary to examine this issue further in future studies.

Various authors have described concentration methods using blood cell separators,[12–17] which typically remove 78%–99% of the red blood cells, while their recovery rates are 78%–82% for mononucleated cells (MNCs) and 83%–100% for CD34+ cells. These results demonstrated that bone marrow cells can generally be collected reliably. These reports also indicate that selective cell transplantation can be performed and that cells can be collected efficiently. However, selective cell transplantation can be performed only at facilities with certain equipment. In addition, even if cell collection can be performed automatically, the process is not simple if one considers the special procedures needed to remove samples from the surgery room. Although the procedure using distilled water is manual, it can be performed on the spot, in a short period of time, without the need for any special device. Therefore, this procedure could find useful applications in clinical practice.

Cells obtained by density gradient centrifugation using a Ficoll solution are mononuclear cells. However, hemolysis using distilled water results in a heterogeneous nucleated cell population that also contains granulocytes. Asahara et al[18] reported that proliferation and differentiation of CD34+ MNCs are more enhanced when cultured in the presence of CD34– MNCs compared with CD34+ MNCs alone. Ghannadan et al[19] reported that VEGF in bone marrow is expressed at high levels in immature cells such as megakaryocytes and myeloid progenitor cells. Kusumanto et al[20] quantitatively examined the localization of circulating VEGF. In healthy volunteers, 34% of the total circulating VEGF was found in platelets and 58% in granulocytes but only 2% in plasma. Minimal VEGF was found in lymphocytes and monocytes. There has also been a report that angiogenic effects of transplanting the entire nucleated cell fraction were the same or better than those of MNC transplantation.[21] Another report indicated that there was no negative effect caused by including cells of the granulocytic series.[1]

Some reports have indicated that secretion of growth factors (such as VEGF, bFGF, and HGF) and cytokines from transplanted bone marrow cells (such asendothelial progenitor cells) was more important than differentiation and proliferation of these cells and their direct contribution to angiogenesis.[3,13,14,22,23] Therefore, angiogenic effects were speculated to be stronger if the entire nucleated cell fraction (including granulocytes) were to be transplanted, compared with selective transplantation of MNCs. In this study, the bone marrow nucleated cell population included not only MNCs but also granulocytes when the cell population was obtained using the hemolysis method with distilled water.

CONCLUSIONS

A clinical application of cell therapy to osteogenesis centers around concentrated bone marrow cells and cultured mesenchymal stem cells have been used widely for animal experiments. In recent years, there have been many reports of increasing the osteogenic ability of cells by using a combination therapy with cultured mesenchymal stem cells and cytokines (such as bone morphogenetic protein-2 and bFGF)[24] and also using modifications of carriers such as hydroxy apatite and βtricalcium phosphate.[25] Our animal experiments used only nonexpanded bone marrow nucleated cells, and the treatments did not include osteogenesis-related cytokines or carrier modifica-

tions for bone induction. Therefore, mature bone formation was not observed histologically, and the healing processes described herein reached only the stage of osteoid formation. Bone formation is an integral part of a complex process involving not only angiogenesis but also spatial and temporal regulation of bone cell proliferation and differentiation. Future studies building on the present results will therefore explore methods that involve administration of other carriers to further enhance the potential clinical benefits of this osmotic hemolytic method.

In other clinical possibilities, the concentration and transplantation of bone marrow cells using distill water method will contribute to the treatment of tissue ischemia, such as critical limb ischemia or compromised flap circulation. This method can be performed in surgery room immediately without any special procedures, such as cell expansion, cell isolation using cell separation medium, or cell separator. It will be a valuable method for clinical use due to its safe and easy characters.

Kazuo Kishi, MD, PhD
Department of Plastic and Reconstructive Surgery
Keio University School of Medicine
35 Shinanomachi, Shinjuku-ward
Tokyo 160–8582, Japan
E-mail: kkishi@sc.itc.keio.ac.jp

REFERENCES

1. Amann B, Luedemann C, Ratei R, et al. Autologous bone marrow cell transplantation increases leg perfusion and reduces amputations in patients with advanced critical limb ischemia due to peripheral artery disease. *Cell Transplant.* 2009;18:371–380.
2. Duong Van Huyen JP, Smadja DM, Bruneval P, et al. Bone marrow-derived mononuclear cell therapy induces distal angiogenesis after local injection in critical leg ischemia. *Mod Pathol.* 2008;21:837–846.
3. Miyamoto M, Yasutake M, Takano H, et al. [Present status of therapeutic angiogenesis by autologous bone marrow cell implantation for refractory chronic peripheral arterial disease and ischemic heart disease]. *J Nippon Med Sch.* 2003;70:436–441.
4. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest.* 2005;115:572–583.
5. Centeno CJ, Kisiday J, Freeman M, et al. Partial regeneration of the human hip via autologous bone marrow nucleated cell transfer: a case study. *Pain Physician* 2006;9:253–256.
6. Hauzeur JP, Gangji V. Phase 1–3 clinical trials using adult stem cells in osteonecrosis and nonunion fractures. *Stem Cells Int.* 2010;26:1–4.
7. Yoshioka T, Mishima H, Akaogi H, et al. Concentrated autologous bone marrow aspirate transplantation treatment for corticosteroid-induced osteonecrosis of the femoral head in systemic lupus erythematosus. *Int Orthop.* 2011;35:823–829.
8. Hendrich C, Franz E, Waertel G, et al. Safety of autologous bone marrow aspiration concentrate transplantation: initial experiences in 101 patients. *Orthop Rev (Pavia).* 2009;1:e32.
9. Gibbs BF, Papenfuss K, Falcone FH. A rapid two-step procedure for the purification of human peripheral blood basophils to near homogeneity. *Clin Exp Allergy* 2008;38:480–485.
10. Fritsch G, Printz D, Stimpfl M, et al. Quantification of CD34+ cells: comparison of methods. *Transfusion* 1997;37:775–784.
11. White WN, Sethu P. Automated microfluidic blood lysis protocol for enrichment of circulating nucleated cells. *J Vis Exp.* 2009;34:1656.
12. Soydan E, Ayyildiz E, Dalva K, et al. Impact of harvest product volume in erythrocyte depletion of allogeneic or autologous bone marrow using COBE spectra. *Transfus Apher Sci.* 2007;36:269–273.
13. Larghero J, Rea D, Esperou H, et al. ABO-mismatched marrow processing for transplantation: results of 114 procedures and analysis of immediate adverse events and hematopoietic recovery. *Transfusion* 2006;46:398–402.
14. Braine HG, Sensenbrenner LL, Wright SK, et al. Bone marrow transplantation with major ABO blood group incompatibility using erythrocyte depletion of marrow prior to infusion. *Blood* 1982;60:420–425.
15. Cassens U, Ostkamp-Ostermann P, Garritsen H, et al. Efficacy and kinetics of bone marrow processing and enrichment of haematopoietic progenitor cells (HPC) by a large-volume apheresis procedure. *Bone Marrow Transplant.* 1997;19:835–840.
16. Witt V, Beiglböck E, Fritsch G. Bone marrow processing with the AMICUS™ separator system. *J Clin Apher.* 2011;26:195–199.
17. Tsubaki K, Ariyama T, Ueno T, et al. Concentration of progenitor cells collected from bone marrow fluid using a continuous flow cell separator. *Ther Apher.* 2001;5:46–48.
18. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–967.
19. Ghannadan M, Wimazal F, Simonitsch I, et al. Immunohistochemical detection of VEGF in the bone marrow of patients with acute myeloid leukemia. Correlation between VEGF expression and the FAB category. *Am J Clin Pathol.* 2003;119:663–671.
20. Kusumanto YH, Dam WA, Hospers GA, et al. Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor. *Angiogenesis* 2003;6:283–287.
21. Hermann PC, Huber SL, Herrler T, et al. Concentration of bone marrow total nucleated cells by a point-of-care device provides a high yield and preserves their functional activity. *Cell Transplant.* 2008;16:1059–1069.
22. Korf-Klingebiel M, Kempf T, Sauer T, et al. Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. *Eur Heart J.* 2008;29:2851–2858.
23. Rehman J, Li J, Orschell CM, et al. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003;107:1164–1169.
24. Akita S, Fukui M, Nakagawa H, et al. Cranial bone defect healing is accelerated by mesenchymal stem cells induced by coadministration of bone morphogenetic protein-2 and basic fibroblast growth factor. *Wound Repair Regen.* 2004;12:252–259.
25. Janicki P, Boeuf S, Steck E, et al. Prediction of in vivo bone forming potency of bone marrow-derived human mesenchymal stem cells. *Eur Cell Mater.* 2011;21:488–507.