Shed Blood-derived Cells from Total Hip Arthroplasty Have Osteoinductive Potential : *A Pilot Study*

Running title: Osteoinduction Using Shed Blood Cells

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Each author certifies that his institution has approved the animal protocol and human cell use and implantation for this investigation and that all investigations were conducted in conformity with ethical principles of research.
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Abstract

Background Cell therapy using autologous cells has been used in the treatment of various medical conditions. The mononuclear cell (MNC) fraction of bone marrow (BM) contains stem/progenitor cells that could contribute to osteogenesis and angiogenesis.
Questions/purposes We asked whether MNCs derived from intraoperative shed blood (SB), consisting of peripheral blood and BM, have osteoinductive and angiogenic potential.
Methods We harvested SB and BM from six patients undergoing THA. Isolated MNCs

from SB and BM were analyzed by flow cytometry to evaluate the CD34+ cell fraction and 1 × 106 cells were seeded on an interconnective porous calcium hydroxyapatite ceramic (IP-CHA) and transplanted in the backs of athymic rats. IP-CHAs without cells were transplanted as controls and all composites were harvested after 4 and 8 weeks. Osteoinductive potential was evaluated by histologic observation, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) using anti-osteocalcin (OC) antibodies qualitatively and quantitatively. To evaluate angiogenic potential, capillary density was measured by immunohistochemistry using Isolectin B4 4 weeks after implantation.
Results We found that CD34+ cells existed in SB-MNCs and there was a trend toward lower frequency compared with BM-MNCs. Histologic osteoinduction, OC expression, and capillary density were increased by transplantation of MNCs from SB. Similar results were achieved with MNCs from BM.
Conclusions MNCs from SB have equivalent osteoinductive and angiogenic potential compared with those from BM.
Clinical relevance SB could be an attractive source for isolation of MNCs, enhancing osteoinduction and neovascularization, to augment the reconstruction of skeletal defects.

Introduction

THA provides long-term pain relief and improved mobility. Nonetheless, revision after hip arthroplasty has constituted close to ¼ of all arthroplasties performed in the United States [14]. Treating bone loss in revision THA remains challenging and some authors advocate grafting of autologous or allogeneic bone [10, 11]. As a result of the limited alternatives for autologous bone and the added morbidity, some surgeons use allogeneic bone, particularly from femoral heads [10]. However, the risk of disease transmission and low osteoinductive potential are disadvantages of allogeneic bone. To address these problems, synthetic biomaterials [18], osteoinductive growth factors [5], and autologous osteogenic cells [4, 16] might provide adequate supplies of pathogen-free biomaterials and improved osteoinduction.

Autologous BM has been proposed as a therapeutic agent for osteoinduction [7, 8, 24]. BM MNCs have been recognized especially as a source of not only hematopoietic/endothelial progenitor cells, but also multipotent stem cells [1, 19]. However, the MNC fraction derived from peripheral blood (PB) also includes hematopoietic progenitor cells [3], endothelial progenitor cells [21], and mesenchymal stem cells (MSCs) [23]. Osteogenic precursor cells reportedly circulate in the PB [6].

Also, endothelial progenitor cells from PB participate in bone formation through vascularization [15].

We therefore asked whether MNCs isolated from intraoperative SB obtained during THA, consisting of PB from bleeding and BM from reaming debris, would have osteoinductive and angiogenic potential.

Materials and Methods

SB and BM were obtained from six patients who underwent THA. SB was collected during surgery and BM was aspirated from the iliac crest. Isolated MNCs from SB and BM were analyzed by flow cytometry to evaluate the CD34+ cell fraction. One million MNCs were resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) and seeded on an IP-CHA. Three experimental groups (six subjects in each group) were created. In the SB group, SB-derived MNCs (SB-MNCs) were seeded on IP-CHA. In the BM group, BM-derived MNCs (BM-MNCs) were seeded on IP-CHA. In the control group, DMEM without cells were seeded on IP-CHA. These composites then were implanted in subcutaneous tissue on the back of the nude rats. All composites were harvested for biochemical and histologic analyses after 4 and 8 weeks (Fig. 1). Osteoinductive potential was evaluated by histologic observation, immunohistochemistry, and ELISA using anti-OC antibodies qualitatively and quantitatively. To evaluate angiogenic potential, capillary density was measured by immunohistochemistry with Isolectin B4 (Vector Laboratories, Burlingame, CA, USA) using sample harvested 4 weeks after implantation. The required sample size was calculated from the estimated difference between the two population mean values of newly formed bone. The newly formed bone area of the control group was assumed to be 5%, and in the experimental group it was assumed to be 70%. When the power of the test was assumed to be 80%, and significant level was assumed to be a one-tailed test on 5%, the necessary size of the sample was 5.71. Thus, we used six subjects. Three composites were implanted in each rat, one for biochemical analysis and two for histologic analyses for bone induction and neovascularization. The institutional animal care and use committee and the human ethics committee of Hiroshima University approved all animal procedures and human cell use and implantation.

We obtained SB and BM from six patients who underwent THA after informed consent (one man, five women; mean age, 58.5 years; range, 51-69 years). SB was collected together with debris from the suction bottle during surgery and BM was aspirated from the iliac crest. Samples of debris and 10 mL BM aspirated from the iliac

crest were treated with 5 mmol/L ethylenediaminetetraacetic acid (EDTA) as an anticoagulant agent and filtered to remove debris. MNCs were isolated using density gradient centrifugal separation using Histopaque 1.077 (Sigma-Aldrich) and washed twice with phosphate-buffered saline after hemolysis with ammonium chloride (StemCell Technologies Inc, Vancouver, BC, Canada).

We used IP-CHA as a scaffold. The IP-CHA was developed by the Department of Orthopaedic Surgery, Osaka University Medical School (Osaka, Japan), Hatano Facility, Covalent Materials Corporation (Kanagawa, Japan), and MMT Company (Osaka, Japan) [22]. This scaffold has the following specific characteristics: porosity, 75%; mean pore diameter, 150 μm; interconnecting pore holes, 10 to 90 μm (mean, 40 μm); and 90% or more of the pores are interconnected through holes. Mechanically, regardless of the porosity being as much as 75%, the compression strength was 10 to 15 MPa because of the uniform arrangement of the pores. For this study, disk-shaped IP-CHA with a diameter of 5 mm and a height of 2 mm were designed and used.

CD34 has been commonly known as a marker of hematopoietic stem/progenitor cells, although some reports [1,15] suggest a CD34+ cell fraction contained not only hematopoietic stem/progenitor cells but endothelial or osteogenic progenitor cells and had angiogenic and osteogenic potentials in vivo. Also, practically, CD45 is expressed on hematopoietic lineages and commonly used for evaluation of CD34+ stem/progenitor cell fraction [1,15]. Based on these data, we presumed CD34+ could be used as one of several markers to evaluate angiogenic and osteogenic potential of cells. We therefore performed flow cytometry using anti-CD34 and CD45 antibodies to evaluate the stem/progenitor cell fraction in BM and SB. For analysis, samples underwent regular flow cytometric profiling with a FACS Calibur analyzer and CellQuestTM Pro software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Dead cells were excluded from the plots on the basis of propidium iodide (PI) staining (Sigma-Aldrich). Cells were washed twice with Hanks' balanced salt solution (HBSS) containing 3.0% heat-inactivated fetal bovine serum (FBS) and incubated with monoclonal antibodies for 30 minutes at 4˚ C after FcR blocking. The stained cells were washed three times with HBSS/3.0% FBS, resuspended in 0.5 mL of HBSS/3.0% FBS/PI, and analyzed. The following monoclonal antibodies were used to identify the CD34+/CD45dim subpopulation, the stem/progenitor cell fraction: CD34-PE (clone 581; Pharmingen, San Diego, CA, USA), CD45-FITC (clone HI30; Pharmingen), IgG1-PE isotype control (Pharmingen), and IgG1-FITC (Pharmingen).

One million MNCs from SB and BM were resuspended in 50 μL DMEM

supplemented with 10% FBS and 1% antibiotics. To seed cells on IP-CHA, cell suspensions were incubated with IP-CHA for 2 hours at 37° C in 5% CO2 before transplantation. IP-CHAs without cells were incubated with DMEM in the same condition and transplanted as controls. SB-MNCs/IP-CHA (SB group) and BM-MNCs/IP-CHA (BM group) and DMEM/IP-CHA (control group) composites were implanted in subcutaneous tissue on the back of the nude rats (F344/N Jcl rnu/rnu; CLEA Japan, Inc, Tokyo, Japan) (female, 7 weeks old, 180-190 g).
Rats were anesthetized with intraperitoneal injections of pentobarbital (Nembutal; Dainippon Pharmaceutical Co Ltd, Osaka, Japan; 1 mL/kg body weight).

After surgery, postoperative pain was managed by administration of subcutaneous injection of buprenorphine hydrochloride (0.2 mg/kg). Rats were kept without antibiotics in the research facilities for laboratory animal science in our university. Four and 8 weeks after implantation, rats were euthanized with an intraperitoneal injection of pentobarbital (2 mL/kg body weight), and composites were harvested for biochemical and histologic analyses.

Using composites harvested 8 weeks after transplantation, we performed histologic evaluations of osteoinductive potential. First, soft tissues surrounding the composites were removed. Composites were fixed in 10% paraformaldehyde and then embedded in paraffin after decalcification with K-CX solution (Fujisawa Pharmaceutical, Tokyo, Japan). Horizontal sections (5 μm) were obtained from the center portion of each composite using a microtome and stained with hematoxylin and eosin (Fig. 2A). The newly formed bone area was measured in five areas of 1.0 mm2, one from the center of the section and four from peripheral areas in the sections using Dynamic Cell Count software BZ-HIC (Model BZ-9000; Keyence, Osaka, Japan) (Fig. 2B). To test the histologic evaluations, three observers (TY, MI, TY) evaluated five areas in sections using hematoxylin and eosin stain and Isolectin B4. Each observer then measured newly formed bone area in the pores and capillary density three times. Between the three observers we found an intraclass correlation coefficient (ICC) of 0.971.

Immunohistochemical staining with anti-rat OC (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) was performed to detect osteocytes in the 8-week composites.

To measure OC concentration in implanted composites, we harvested composites from each group 4 and 8 weeks after implantation. We homogenized composites in tissue protein extraction reagent (Thermo Fisher Scientific, Inc, Kanagawa, Japan) containing protease inhibitor, and homogenates were centrifuged at 15,000 rpm for 5 minutes at 4˚ C. The supernatants were collected and duplicates assayed for OC concentration with an ELISA for rat OC (GE Healthcare Japan, Tokyo, Japan). The

protein concentrations of the lysates were adjusted to 0.1 μg/mL, and 100 μL of the lysates was used to measure OC content with a microplate reader at an absorbance of 450 nm.

To evaluate angiogenic potential, composites at 4 weeks were fixed and decalcified with 0.5 mol/L EDTA and sectioned as described previously. The central sections of composites 4 weeks after implantation were stained with Isolectin B4 [15]. Capillaries were recognized as blood vessels with endothelium-positive for Isolectin B4. The number of capillaries was counted in five areas of 1.0 mm2 in each composite following the manner described previously. The mean of the number of capillaries in five areas was termed the capillary density.

We compared the percentages of CD34+ cells in BM and SB using the Mann-Whitney test.

The Kruskal-Wallis test was performed to examine differences between the three groups and then a post hoc test was performed using Dunn's multiple comparison test. Group mean values and standard deviations were calculated, and the results are reported as means ± standard deviation.

Results

MNCs isolated from BM and SB contained CD34+ cells. We observed no difference in the percentage of CD34+ cells in BM than SB (Fig. 3).

Eight weeks after implantation, hematoxylin and eosin staining showed bone formation in the pores of composites, which had been seeded with MNCs from SB or BM (Fig. 4) In contrast, control composite implants had only fibrous tissue formation (Fig. 4A). Greater bone formation was induced by BM-MNCs and SB-MNCs compared with controls. The mean area of newly formed bone was similar in composites seeded with BM or SB (Fig. 4D).

Using immunohistochemical staining with antirat OC, lamellar bone and OC-positive osteocytes were observed in the pores of scaffolding seeded with BM and SB after 8 weeks (Fig. 5A-C). The expression level of OC was greater in the BM and SB groups than in the control group at 4 and 8 weeks after implantation, but we observed no difference between BM and SB at either time (Fig. 5D).

Composite samples 4 weeks after implantation stained with Isolectin B4 showed greater capillary densities in the BM and SB groups than in the control group (Fig. 6).

Discussion

BM-MNCs have been recognized as a source of not only hematopoietic/endothelial

progenitor cells, but also multipotent stem cells [1, 19]. However, PB-derived MNC also includes hematopoietic progenitor cells [3], endothelial progenitor cells [21], and MSCs [23]. Furthermore, osteogenic precursor cells reportedly circulate in the PB [6] and endothelial progenitor cells from PB participate in bone formation through vascularization [15]. Therefore, we asked whether intraoperative SB, which includes MNCs from PB and BM, should contain cells with osteoinductive and angiogenic capacities.

The current study has several limitations. First, in previous reports, engraftment of transplanted human cells was evaluated by immunofluorescent staining using anti-human nuclear or mitochondria antibodies [9, 15]. Immunofluorescent staining with human-specific antibody generally requires frozen sections, which have not been decalcified. However, because of the mechanical properties of IP-CHA, we could not obtain satisfactory sections without decalcification. Consequently, we observed osteoinduction and neovascularization induced by cell implantation in nude rats using decalcified samples. As a result, it is not clear whether transplanted SB-derived human cells differentiated to osteocytes or endothelial cells or whether they supported the growth of host rat cells. Second, our flow cytometric data showed that CD34+ cells existed in SB-MNCs and their frequency was not statistically different compared with that of BM-MNCs, although there was a lower trend. However, our limited in vitro experiments showed angiogenic and osteoinductive potentials of SB-MNCs that contained CD34+ cells, not those of purified CD34+ cells. Therefore, it would be important to determine whether and how CD34+ cells could contribute to osteoinduction and angiogenesis. Third, for one of the methods used to evaluate osteoinduction, we performed immunohistologic staining using anti-OC antibody and showed the osteoinductive potential of SB-MNCs qualitatively. However, to rigorously explore osteoinduction it would be important to determine the effects of these cells on calcium deposition, alkaline phosphatase activity, and image analysis. Fourth, we did not determine the number of cells isolated from SB. In further studies, the volume-cell salvage yield from SB in THA or revision THA should be evaluated. As a rule, SB usually has been suctioned and discarded as clinical waste unless erythrocytes were collected for retransfusion. Minimizing the amount of SB is desirable for homeostasis of the patient. Therefore, our main focus was not to maximize the number of cells from SB, but rather to confirm that recoverable autologous osteoinductive and angiogenic cells are present. Fifth, we incubated MNCs with IP-CHA to seed the composite. However, we have not evaluated optimal conditions for osteoinduction and neovascularization. We intended to simulate clinical conditions as previously described

Although a longer culture span might accelerate osteoinductive potential as shown in MSCs [12], we plan to isolate the osteoinductive source from SB during surgery without culture that usually requires special devices and facilities. Therefore, to minimize the time for hybridization of the cells on the composite will be an important factor for clinical application and further study is required. Finally, the physical size of scaffolds and the biological environment in the subcutaneous tissues of the back in this rat model could be different from that of human skeletal defects in clinical situations. In addition, we have not evaluated optimum number of MNCs required for effective osteoinduction considering various sizes of defects. From these points, we think additional studies are required using a more appropriate model simulating clinical conditions and validating the relationship between cell number and defect size.

MSCs have promising osteogenic potential in clinical settings [13, 17]. However, Porter et al. developed a special device to collect reaming debris during hip arthroplasty as a source of MSCs [20] and showed MSCs could be isolated and differentiated to osteogenic, adipogenic, and chondrogenic lineages in vitro. Our approach however, was to collect intraoperative SB containing PB and reaming debris. Although the MSC-containing fraction might be diluted by PB, our data suggest SB-MNCs have osteoinductive potential similar to that of cells isolated from reaming debris.

In addition, our data suggested that SB-MNCs have angiogenic potential similar to that of BM-MNCs by capillary densities. Matsumoto et al. reported that endothelial progenitor cells from PB participated in bone formation through vascularization [15]. From this evidence, there is a possibility that MNCs from SB containing PB and BM contributed to bone formation through promotion of vascularization similarly.

Ogushi et al. described ectopic bone formation using porous calcium phosphate ceramic combined with autologous BM cells in a rat model [16]. From their data, the process of bone formation consisted of membranous ossification without cartilage formation beginning at approximately 3 weeks. They then reported mesenchymal cells cultured from freshly isolated BM accelerated bone formation in the porous regions of ceramic as early as 2 weeks after implantation [25]. Although transplantation of MSCs, which are more committed to the osteogenic lineage, is a desirable option to induce osteogenesis in vivo, the cultivation process could add risks such as contamination and technical complications in clinical settings.

Autologous BM-MNCs have been used for several clinical treatments such as peripheral occlusive arterial disease, osteonecrosis of the femoral head, and coronary surgery [2, 7, 8, 26]. Yamasaki et al. reported that BM-MNCs can be implanted in patients being treated for osteonecrosis of the femoral head [24]. This approach

promotes bone regeneration in the necrotic region [24]. In these therapies, isolation of MNCs usually is performed simultaneously with the blood centrifugation system in the same operating room. From these backgrounds, we speculated that SB-MNCs could be a novel cell source although there are several problems to be solved such as sterile processing, coagulopathy, fibrinolysis, and platelet dysfunction. Our data suggest that cells from SB, usually discarded as clinical waste, could be a practical option to enhance osteoinduction and neovascularization.

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Legends
Fig. 1 A flow diagram of the experiments (cell preparation and in vivo transplantation) is shown. Bone marrow (BM) and shed blood (SB) are harvested from patients during THA.
Isolated mononuclear cells (MNCs) (1 × 106) from BM and SB are seeded on interconnective porous calcium hydroxyapatite ceramic (IP-CHA) and transplanted in the backs of athymic rats for one biochemical and two histologic analyses. IP-CHAs without cells were transplanted as controls. There were three groups and six subjects per group in this study.
Fig. 2A-B (A) Composites (disk-shaped with a diameter of 5 mm and a height of 2 mm) were sectioned (5 μm) horizontally using a microtome and the center portion of the composite was stained. (B) The newly formed bone and capillary were assessed in five areas (one center and four peripheral per section) of 1.0 mm2.
Fig. 3A-B (A) The frequencies of CD34+/CD45dim cells were evaluated in the bone marrow (BM) and shed blood (SB) groups. A dot plot shows the representative flow cytometric analysis. (B) The percentage of CD34+/CD45dim cells in SB showed lower tendency compared with BM. However, there was no difference between the BM and SB groups (BM group, 2.86% ± 1.03%; SB group, 2.42% ± 0.82%; p = 0.6991 for BM versus SB).
Fig. 4A-D (A-C) Histologic observation revealed fibrous tissues and bone formation in composites harvested at 8 weeks after implantation in each group. In the (A) control group, only fibrous tissues were seen although substantial bone formations in the pores of the composite were identified in the (B) bone marrow (BM) and (C) shed blood (SB) groups. (H = interconnective porous calcium hydroxyapatite ceramic (IP-CHA); F =

fibrous tissue; L = lamellar bone) (A-C: Stain, hematoxylin and eosin; original magnification, x400; scale bar = 100 μm). (D) A graph shows the percentage of newly formed bone in the composite 8 weeks after implantation. Compared with the control group, the percentage was higher in the BM and SB groups (control group, 10.4% ± 6.7%; BM group, 26.8% ± 4.9%; SB group, 25.9% ± 3.1%; p = 0.0150 for control versus BM; p = 0.0065 for control versus SB; p = 1.0000 for BM versus SB).

Fig. 5A-D Lamellar bone and lining osteocalcin (OC)-positive osteocytes are identified in the bone marrow (BM) and shed blood (SB) groups 8 weeks after implantation in the (A) control, (B) BM, and (C) SB groups. [H = interconnective porous calcium hydroxyapatite ceramic (IP-CHA); F = fibrous tissue; L = lamellar bone, white arrows: lining OC-positive osteocytes](Stain, immunochemical stain; original magnification, x400; scale bar = 100 μm). (D) Four and 8 weeks after implantation, the expression level of OC was higher in the BM and SB groups compared with the control group. There was no difference between the BM and SB groups at either time (4 weeks: control group, 18.6 ± 3.5 ng/mL; BM group, 36.8 ± 3.2 ng/mL; SB group, 39.5 ± 9.0 ng/mL; p = 0.0065 for control versus BM; p = 0.0260 for control versus SB; p = 0.3961 for BM versus SB) (8 weeks: control group, 22.2 ± 10.9 ng/mL; BM group, 50.0 ± 6.5 ng/mL; SB group, 49.4 ± 19.2 ng/mL; p = 0.0065 for control versus BM; p = 0.0455 for control versus SB; p = 1.0000 for BM versus SB).

Fig. 6A-D Capillaries were identified as blood vessel endothelium positive for Isolectin B4 in the (A) control, (B) bone marrow (BM), and (C) shed blood (SB) groups (Stain, immunohistochemical stain; original magnification, x400; scale bar = 100 μm). (D) The density of capillaries was greater in the BM and SB groups compared with the control group at 4 weeks. There was no difference between the BM and SB groups (control group, 104 ± 7.1/mm2; BM group, 215 ± 19.4/mm2; SB group, 217 ± 25.5/mm2; p = 0.0150 for control versus BM; p = 0.0150 for control versus SB; p = 1.0000 for BM versus SB).

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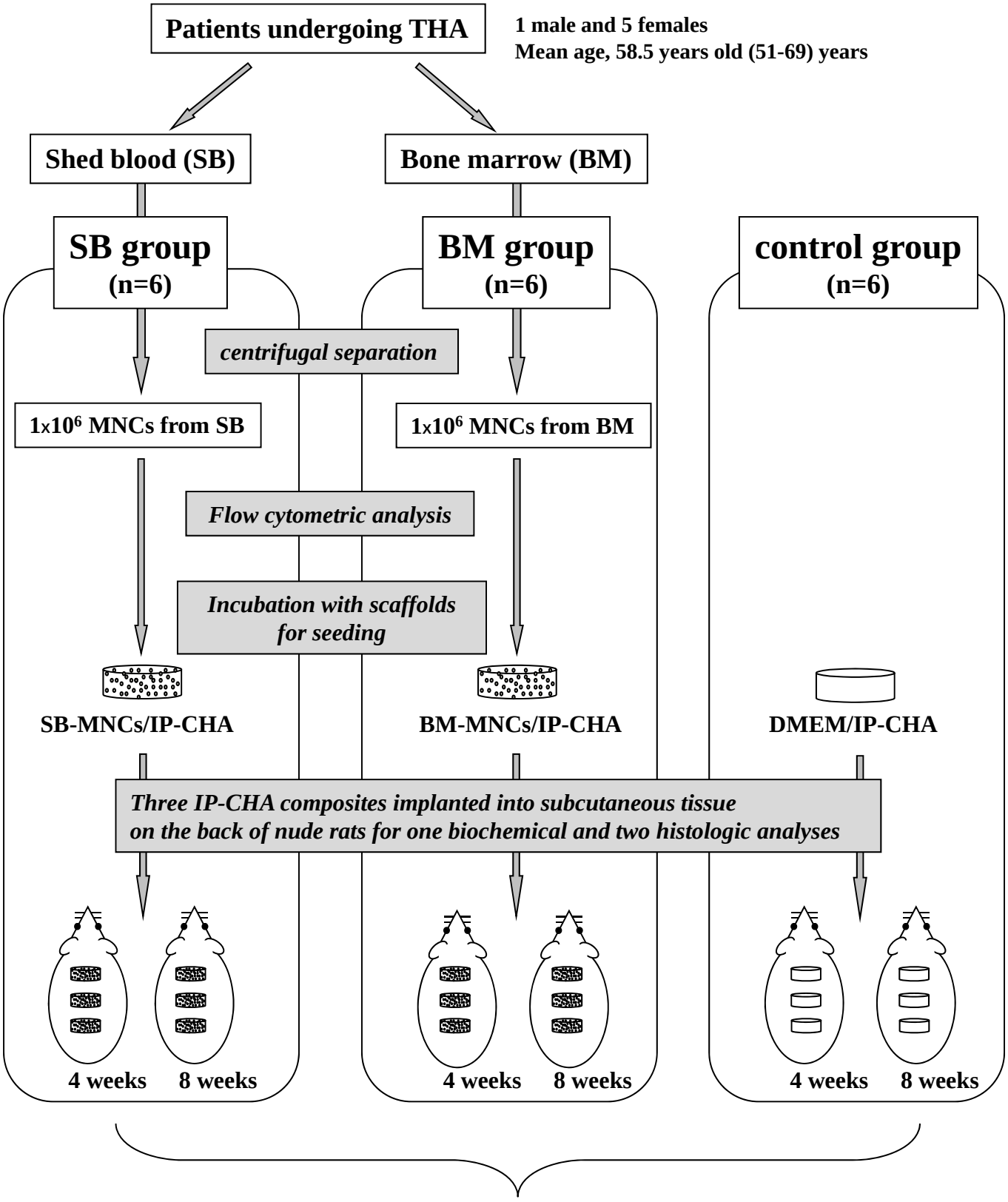
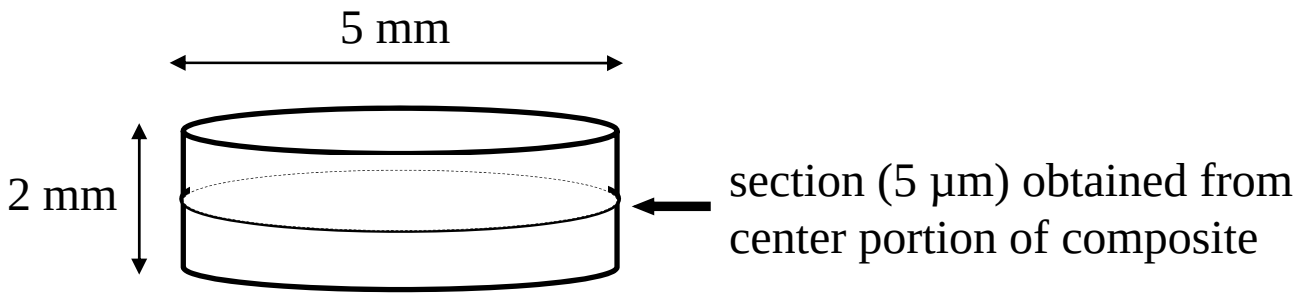
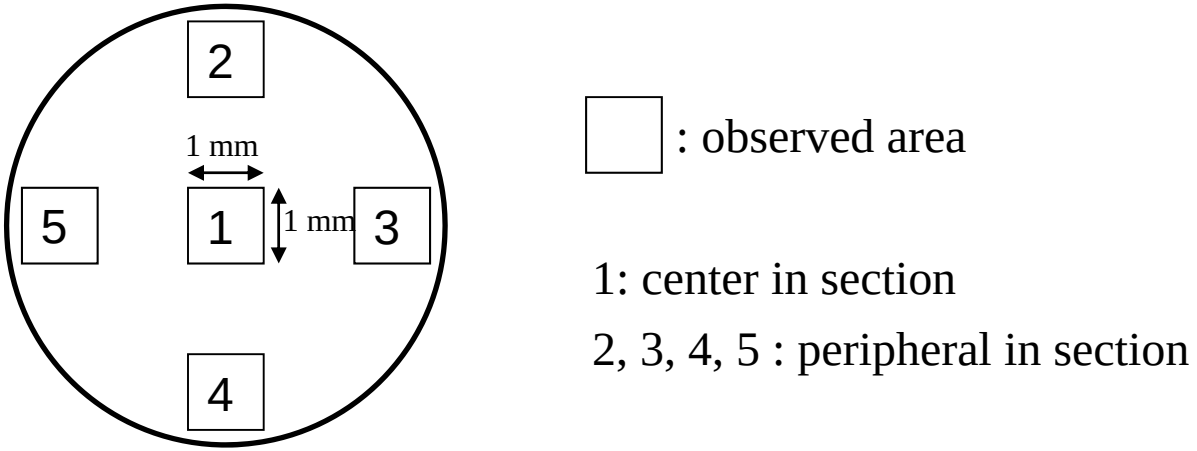


IP-CHA composites were harvested for biochemical and histologic analyses
4 and 8 weeks after implantation

Fig. 2

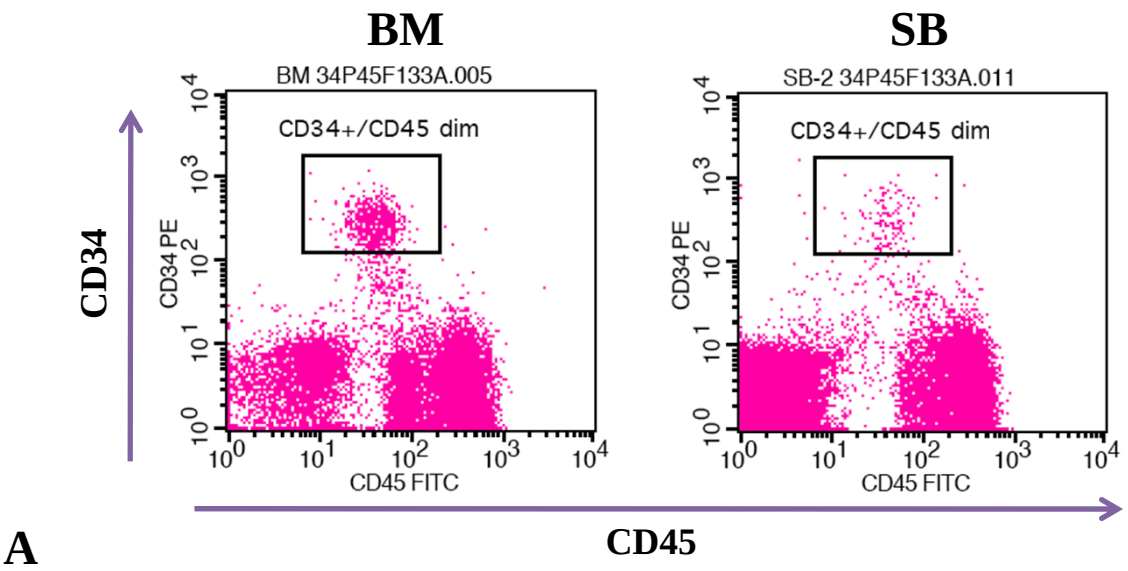


A

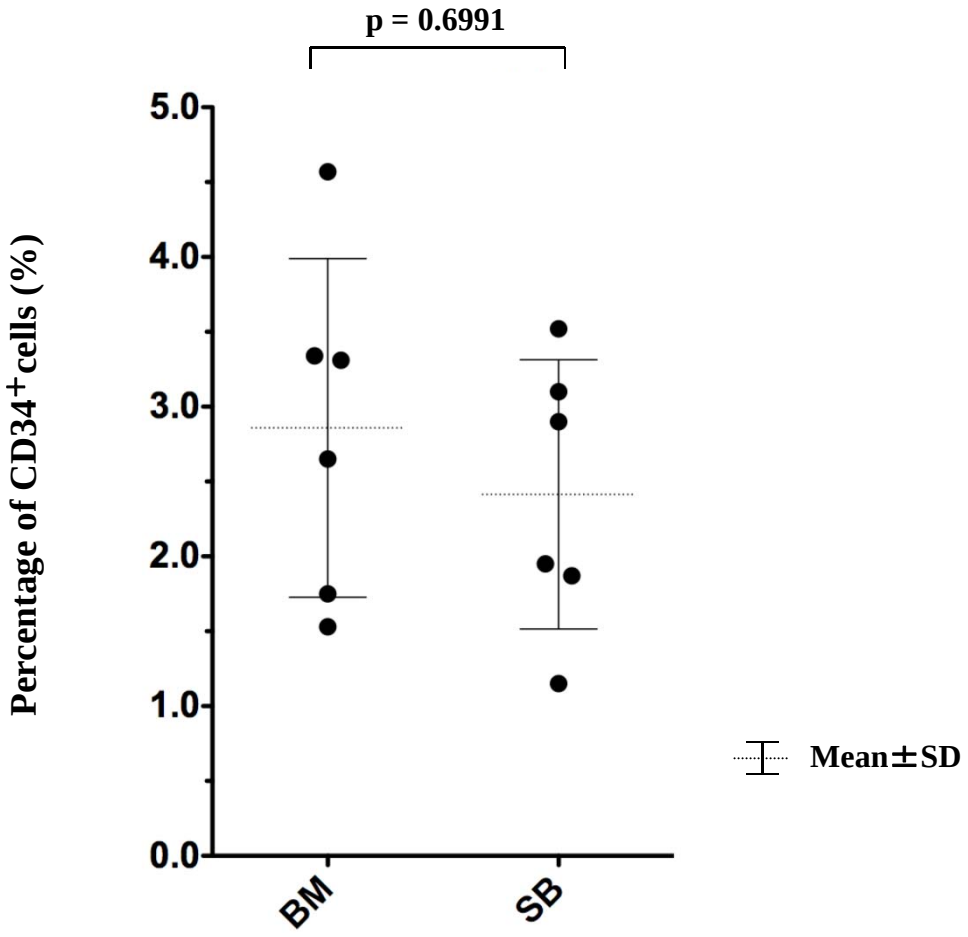


B section (5 μm) from center portion

Fig. 3

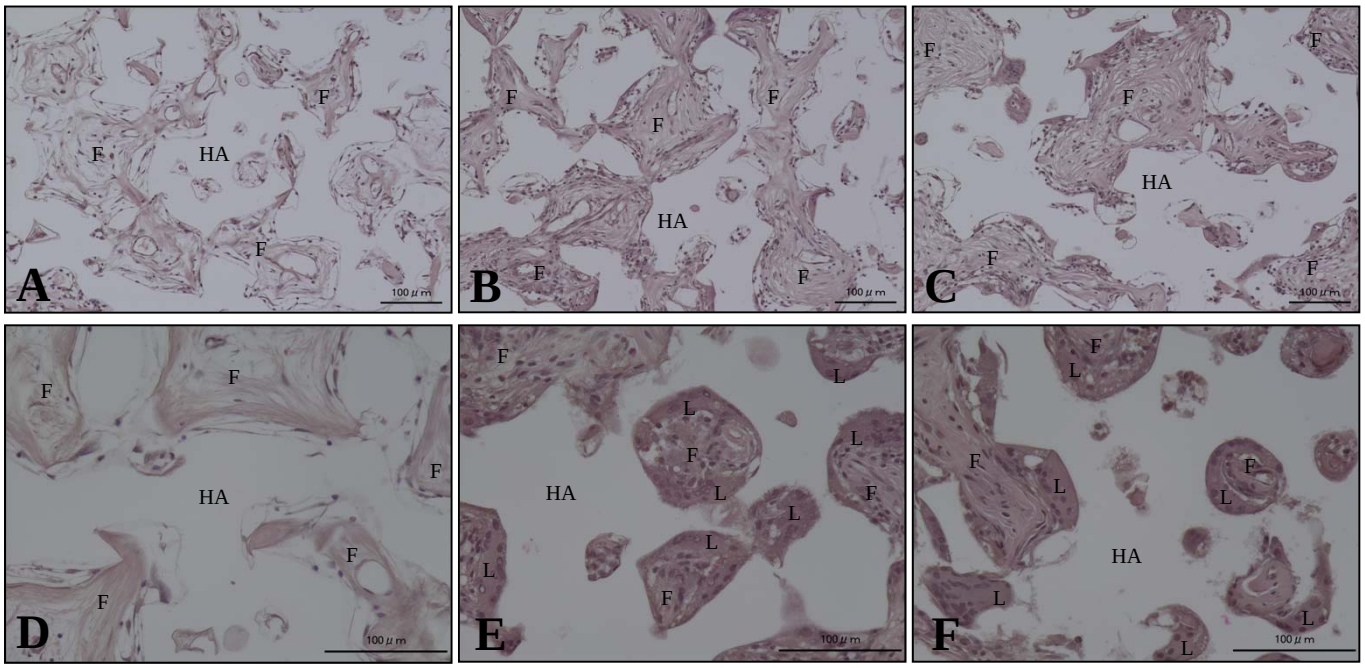

BM
SB
BM 34P45F133A.005
SB-2 34P45F133A.011
CD34+/CD45 dim
CD34+/CD45 dim
CD34 PE
CD45 FITC
CD34
CD45

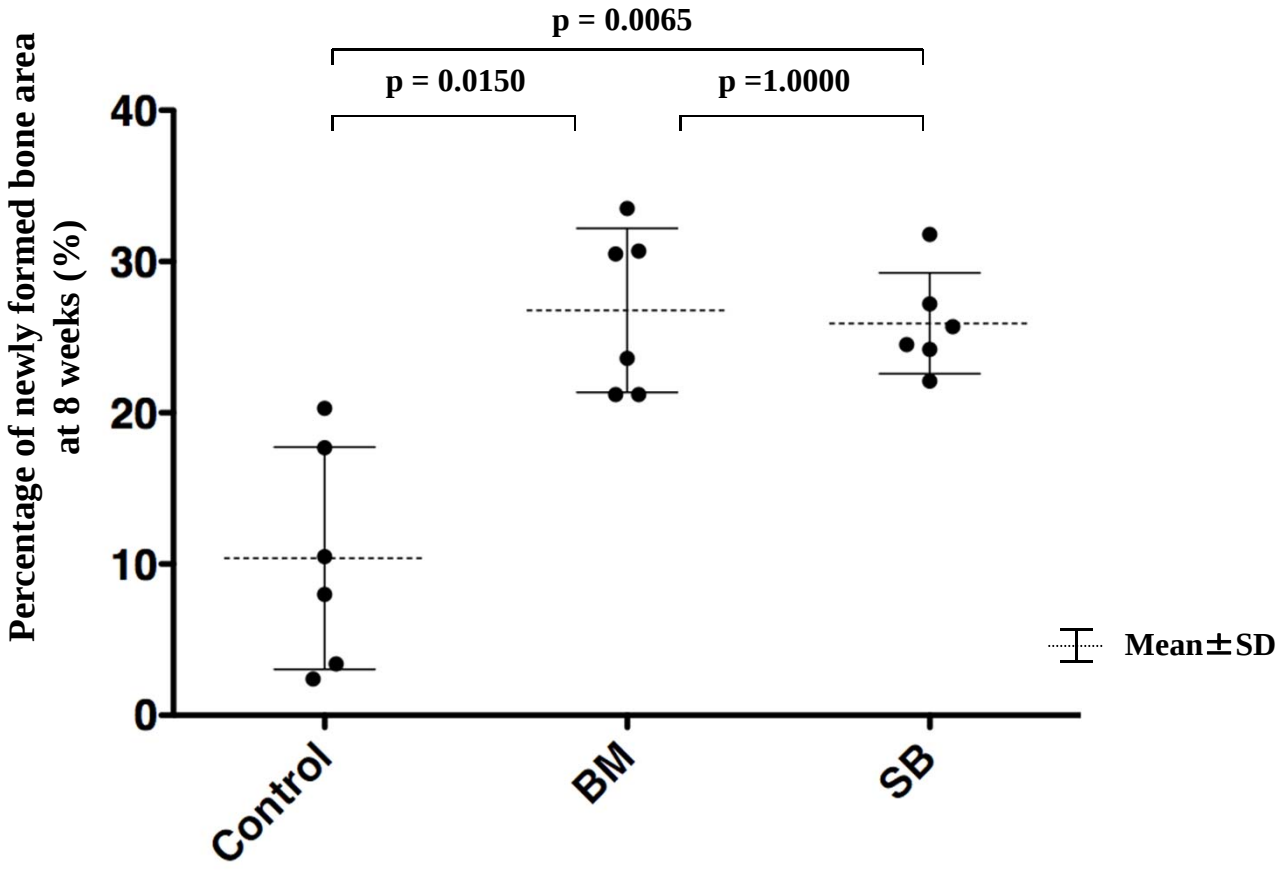
A


p = 0.6991
Percentage of CD34+cells (%)
5.0
4.0
3.0
2.0
1.0
0.0
BM
SB
Mean±SD

B

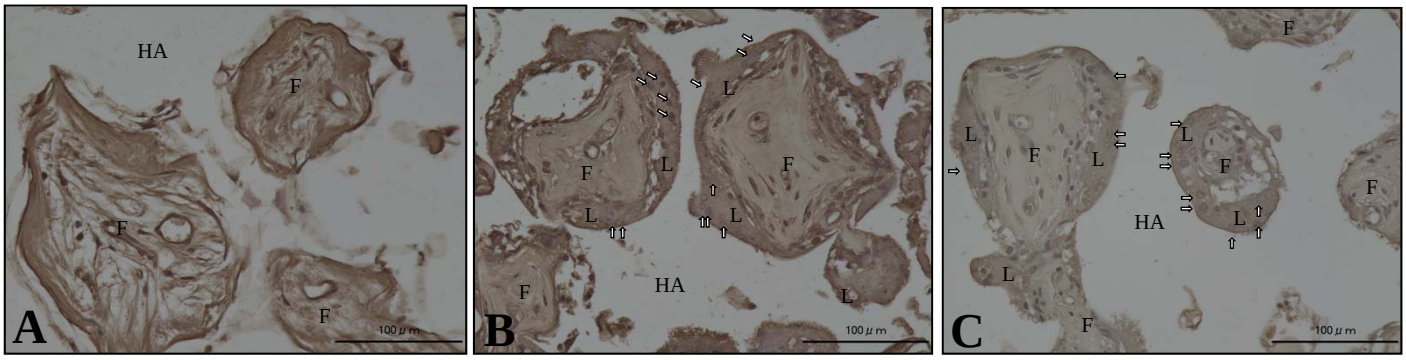
Fig. 4A-G

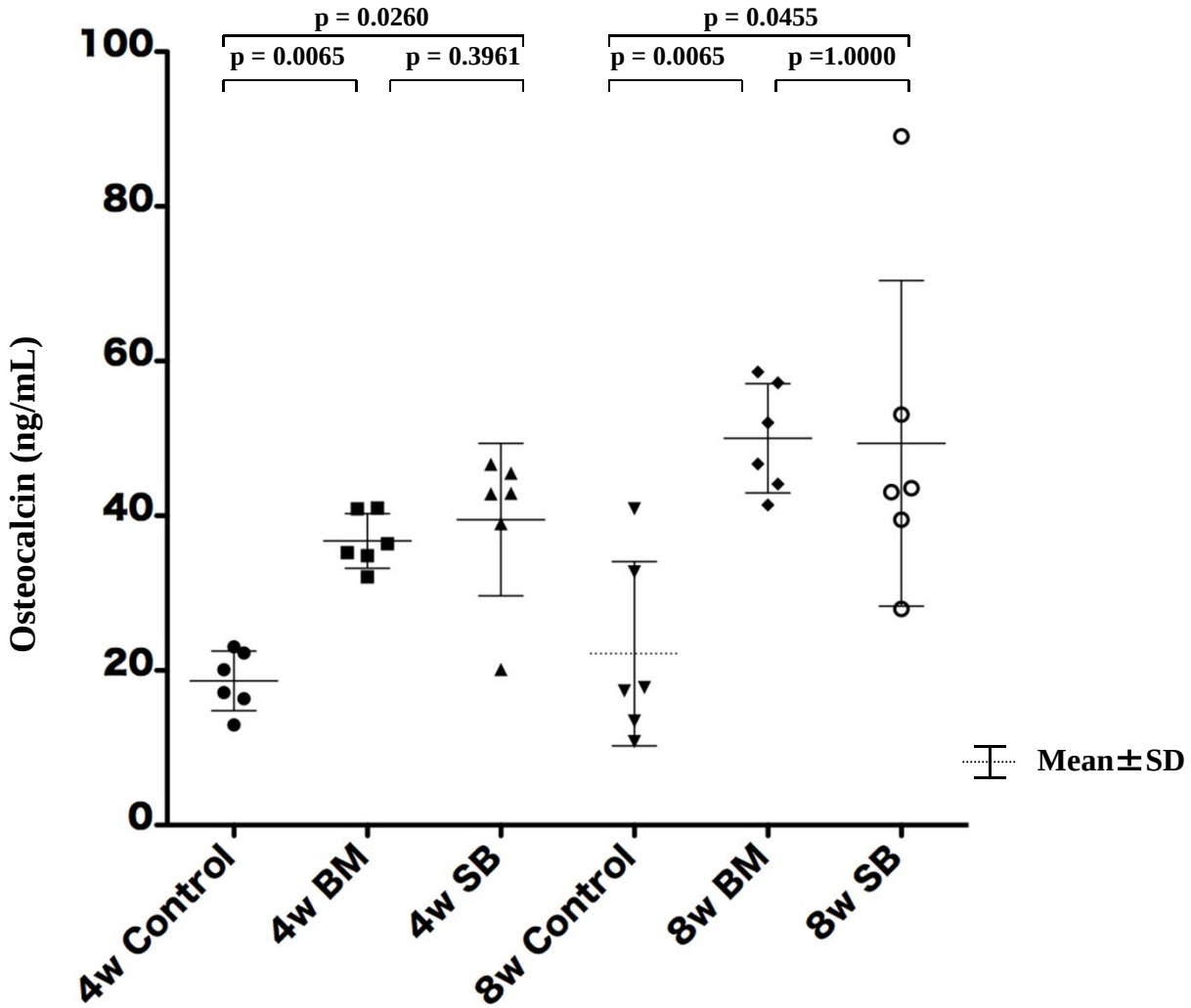

A
B
C
D
E
F
HA
F
L
100 μm


p = 0.0065
p = 0.0150
p =1.0000
Percentage of newly formed bone area at 8 weeks (%)
40
30
20
10
0
Control
BM
SB
Mean±SD

G

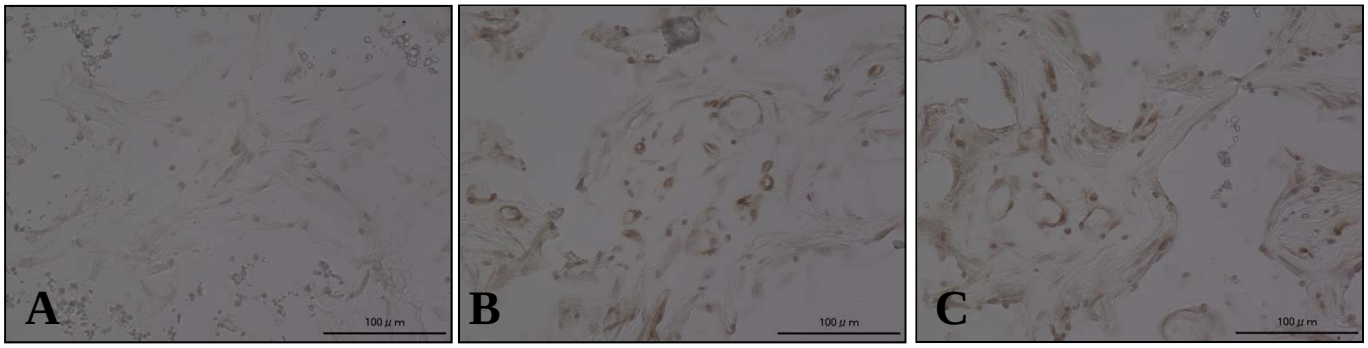
Fig. 5A-D

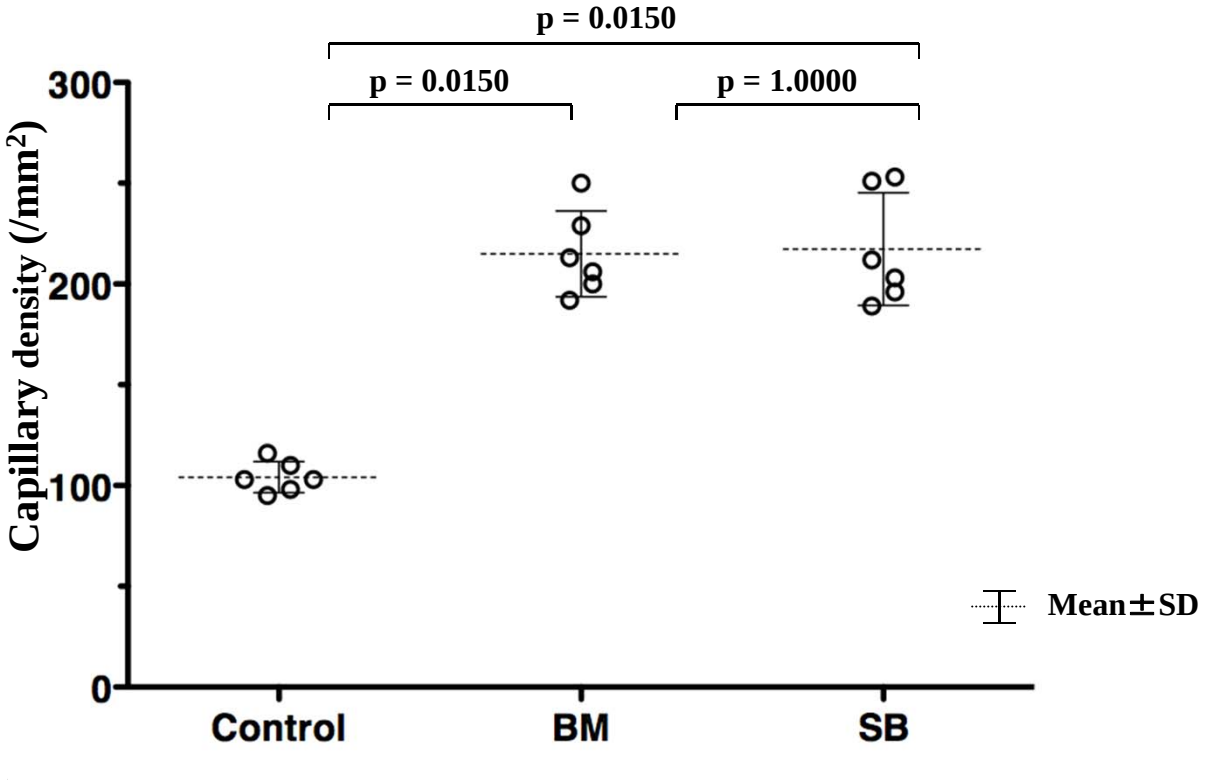

HA
F
L
A
B
C
100 μm


p = 0.0260
p = 0.0065
p = 0.3961
p = 0.0455
p = 0.0065
p =1.0000
Osteocalcin (ng/mL)
100
80
60
40
20
0
Mean±SD
4w Control
4w BM
4w SB
8w Control
8w BM
8w SB

D

Fig. 6A-D


A
100 μm
B
100 μm
C
100 μm


p = 0.0150
p = 0.0150
p = 1.0000
Capillary density (/mm²)
300
200
100
0
Control
BM
SB
Mean±SD

D