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Bone regeneration of double-canaled implant with peripheral blood derived mesenchymal stem cells: *in vitro* and *in vivo*

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-Abstract-

Bone regeneration of double-canaled implant with peripheral blood derived mesenchymal stem cells: *in vitro* and *in vivo*

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Purpose: Peripheral blood (PB) is known as a source of mesenchymal stem cells (MSCs) like bone marrow (BM). It is easy to get, however, it has limited proved that the osteogenic differentiation potential of PB-derived MSCs after implant in dentistry. In this study, we characterized rabbit PB-derived MSCs compared to BM-derived MSCs *in vitro* and *in vivo*. Furthermore, we proved the osteogenic ability in the process of double-canaled implant surgery model of rabbits.

Materials and methods: After collection and purification of PB and BM from New Zealand white rabbit, we isolated PB- and BM- derived MSCs. PBMSCs (peripheral blood mesenchymal stem cells) were cultured on the extracellular matrix (ECM)-coated culture plate which was made by BMMSCs and used to obtain the PBMSCs. BMMSCs (bone marrow mesenchymal stem cells) were cultured on the plastic culture plate, and used as a control. For characterization of PBMSCs, we performed morphology study, fluorescence-activated cell scanner (FACS) to

analyze positive expression of MSC surface molecular CD14 and CD90, cell proliferation assay, and multiple differentiation assay *in vitro* comparing with BMMSCs. And then the PBMSCs were mixed with hydroxyapatite/tricalcium (HA/TCP), transplanted into immunocompro-mised mice (BALB/c), and stained with hematoxylin and eosin (H&E) to identify the osteogenic ability of the PBMSCs. For bone regeneration of the PBMSCs in double-canaled implant, HA/TCP, mix of PBMSCs and HA/TCP, and mix of BMMSCs and HA/TCP were applied to double-canaled implant, but defect double-canaled implant were not filled. During the installation into rabbit tibia, the upper and lower canal of implant were located in the cortical and marrow area respectively. After 3 weeks and 6 weeks, the sectioned implants were stained with toluidine blue and von kossa for histological examination and histomorphometric analysis.

Results: The PBMSCs showed fibroblastic-like morphology, similar rate of BrdU positive cells to BMMSCs, and positively expressed CD90 (~31.8%), but negatively showed CD14 (~0.9%). For osteogenic differentiation, nodules were stained with red and black by Alizarin red S and von kossa, respectively. Lipid droplets were detected by Oil red O staining for adipogenic differentiation, and cartilage matrices were observed by Alcian blue and Safranin O staining for chondrogenic. After transplantation *in vivo*, the PBMSCs generated bone which were stained with H&E. In result, there was no difference bone formation area between PBMSCs (~41%) and BMMSCs (~43%). Histological examination of the double-canaled implant in PBMSCs and BMMSCs showed more mature bone, which were stained with toluidine blue and von kossa in both the upper and lower canals at 6 weeks and in the upper

canal at 3 weeks. Histomorphometric analysis results proved that the PBMSCs (~16%) and BMMSCs (~17%) groups showed higher new bone (NB) than groups of HA/TCP (~12%) and defect only (~10%) in the upper canal at 3 weeks, however there was no different results of NB among all groups (defect only: ~3.7%, HA/TCP: ~4.8, BMMSCs: ~ 4.9%, PBMSCs: ~4.5%) in the lower canal. At 6 weeks, groups of the PBMSCs (upper canal: ~36%, lower canal: ~8%) and BMMSCs (upper canal: ~35%, lower canal: ~7.2%) showed higher NB than the HA/TCP (upper canal: ~21%, lower canal: ~6.0%) and defect only groups (upper canal: ~15%, lower canal: ~5.0%) in both the upper and lower canals.

Conclusions: The PBMSCs have characteristics and bone regeneration ability just like BMMSCs, not only *in vitro* but also *in vivo.* ECM was effective of obtaining PBMSCs. Therefore, the PBMSCs could be the promising source for bone regeneration in dental clinical uses.

Key words: Bone marrow stromal cells, peripheral blood, mesenchymal stem cell transplantation, dental implantation, matrix, extracellular

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CONTENTS

Ⅰ. Introduction

Mesenchymal stem cells (MSCs) represent an excellent cell source for cell-based bone regeneration due to their capacity of giving rise to different cell lineages including osteocytes, adipocytes, and chondrocytes[1]. MSCs were isolated from a variety of tissues, but generally bone marrow (BM) is regarded as the richest and the major source of MSCs. Bone marrow mesenchymal stem cells (BMMSCs) have been considered as an alternative source because they could regenerate bone defect in several animal models[2]. Nevertheless, the method of

aspiration BM from patients is highly invasive procedure, which may aggressive to the donor sites of patients[3]. In contrast, peripheral blood (PB) appears to be an alternative and more readily available source of MSCs, because the isolation of PB is minimally invasive without any complications, and does not require further general anesthesia compared with BM[4]. It would be very useful if MSCs harvested from PB are expanded to enough numbers with their osteogenic capacity. Since MSCs were isolated from PB, several distinct progenitor cell populations have been reported in the peripheral blood mononuclear cells (PBMNCs), including hematopoietic stem cells (HSCs)[5], endothelial progenitor cells (EPCs)[6], mesenchymal stem cells (MSCs)[7], osteoclast precursor cells[8], hematopoietic osteoclast precursor cells[9] and circulating fibrocytes[10], which suggest that PBMNCs may possess a potential to differentiate multitude functional cell types in specific microenvironment. There were some evidences that increasingly number of MSCs were released into PB under specific pathological conditions, which illustrated that MSCs could be involved into tissue repair taken PB as a pathway of BM homing to damaged tissues[11-13]. However, it remains unclear and controversial whether MSCs could be isolated from PB under normal conditions. At present, the most common method of isolating MNCs from PB is density gradient centrifugation over ficoll-paque for basic research use. After isolating the PBMNCs, specific progenitor cells (HSCs, MSCs, and EPCs) can be purified. Kim and colleges selected the MSCs from PBMNCs via simply discarding the non-adherent cells 48 hours after first primary seeding[14]. Fibrin matric was also proposed to MSCs culture according to their highly binding efficiency[15]. However, both the quantity and quality of the MSCs are still dissatisfied with appliance. Therefore, the effective

method of isolation MSCs from PB is significant valuable for cell-based bone regeneration.

Recently, it was reported that extracellular matrix (ECM) promoted the proliferation of MSCs and facilitated the control of MSCs fate. ECM containing collagens, fibronectin, adhesive proteins, and molecular weight proteoglycans favors the proliferation, self-renewal, and multi-potential differentiation, suggesting that ECM secreted by MSCs modulates cell behavior and facilitates retention of MSCs properties[16]. ECM plays a critical role in the control of MSCs behavior has been indicated by previous study, which showed that lacking the biglycan exhibits deficiency in the ability of the MSCs to differentiate into osteogenesis. In our previous study, ECM deriving from BMMSCs was valuable for propagation of the suspended cells in rabbit model[17]. The suspended cells obtained by ECM-coated culture plates had the similar characteristics and bone regeneration capacity as like those of BMMSCs[17]. Thus, we hypothesized that the ECM has an important component of controlling MSCs, and mobilizing PBMSCs could be acquired with ECM. In this study, we endeavored to acquire the MSCs from PB of rabbits using the method of culturing on the ECM, and identified the characters of the PBMSCs *in vitro* and *in vivo.* When MSCs are acquired from PB using the method of culturing on the ECM, it would be very convenient to clinical use for patients in future. Also, if the MSCs from PB have the similar characters to the BMMSCs, it would be considered as a useful MSCs source for cell-based bone regeneration.

Bone regeneration is a complicated process and involves different steps in the cortical and marrow areas of a surgical site. The easiest

method for the study of bone regeneration is to prepare a defect in the bone, locate the substitute on bone defect area, and retrieve the regenerated are of the bone tissue. However, this method is not a good application because of the difficulty in locating the defect as bone replacement which should maintain mechanical stability and volume during healing period. The double-canaled implant used in this study is well-established model to evaluate the bone regeneration in different areas of bone[18]. The double-canaled implant is simply to prepare at the same time. The double canal also can be filled with various bone grafting materials and be served as a space of graft materials for bone regeneration evaluation. Pervious study have been reported that the double-canaled implant was valuable for applying the different graft materials for bone regeneration[18]. Our previous study also reported that the double-canaled implant considered to be a useful model for applying the suspended cells to the bone regeneration at rabbit[17]. We investigated the capacity of the bone regeneration in different areas of the bone by applying the acquired PBMSCs to the double-canaled implant.

The aim of this study was to acquire the MSCs from PB of rabbit, identify characteristics of the PBMSCs, and recognize possibility of the PBMSCs for bone regeneration in the double-canaled implant model at rabbit.

Ⅱ. Materials and methods

1. Acquisition of the PBMSCs from rabbit

1-1. PBMSCs preparation and culture

This experiment using New Zealand white rabbits were approved by the Animal care and use committee of Dentistry of Seoul National University and carried out in accordance with the Guidelines and Regulations (Approval number: SNU-130312-1). PB was obtained before BM aspiration during the general anesthesia. 10 ml of PB samples were collected into anti-coagulant tube from the marginal ear vein in a sterile manner, placed on ficoll-paque density gradients, and centrifuged at 400g for 30 min. The middle layer containing MNCs were transferred to a 50ml tube and washed with phosphate buffered saline (PBS). The collected MNCs were treated with the Ammonium-Chloride-Potassium (ACK) lysing buffer (Lonza, MD, USA) for 5 min at room temperature (RT). The purified MNCs were suspended in ECM-coated culture plates with basic culture medium consisting of alpha-modified eagle's medium (α-MEM) (Life Technologies, NY, USA) supplemented with 15% fetal bovine serum (FBS) (Equitech-Bio, TX, USA), (2mM) Glutamine, (55 μM) 2-Mercaptoethanol, Penicillin/streptomycin (Life Technologies, NY, USA), and (100 μM) L-ascorbic acid (Wako pure, Tokyo, Japan) and cultured in a humidified atmosphere at 37℃ with 5% CO_2. The cells were trypsinized on plastic culture plates 14 days later for the following experiments and the medium was changed twice weekly (Fig. 1).

1-2. BMMSCs preparation and culture

The procedure of rabbit BM aspiration and culturing was described as our previously study[17]. BM aspirations were taken from rabbit tibia under general anesthesia using ketamine (Yuhan, Gunpo, Korea) and rompun (Bayer Korea, Ansan, Korea). The skin and cortical bone areas of the tibia were punctured with a BM aspirator (Unimed SA, Lausanne, Switzerland) under local anesthesia with the lidocaine (Yuhan, Gunpo, Korea) containing 1:100000 epinephrine. After confirming that the aspirator was fixed, 8 to 10ml of BM samples were aspirated with a 22 gauge syringe containing 0.5ml of the heparin (Choongwae Pharm, Seoul, Korea). The aspiration samples were diluted 1:1 with PBS, filtered through a 70mm nylon mesh filter (Becton Dickinson, NJ, USA), loaded onto an equivalent volume of ficoll-paque density gradients, and isolated by density gradients centrifugation at 400g for 30 min. The collected MNCs were treated with the ACK lysis buffer (Lonza, MD, USA) for 5 min at RT. The purified MNCs were suspended in plastic culture plate (Corning, NY, USA) with basic culture medium consisting of α-MEM (Life Technologies, NY, USA) supplemented with 15% FBS (Equitech-Bio, TX, USA), (2mM) Glutamine, (55μM) 2-Mercaptoethanol, Penicillin/streptomycin (Life Technologies, NY, USA), and (100μM) L-ascorbic acid (Wako pure, Tokyo, Japan) and cultured in a humidified atmosphere at 37℃ with 5% CO_2 for 14 days. (Fig. 1). The binding cells were sub-cultured for following experiments.

1-3. ECM-coated culture plate preparation

For reconstituting of ECM-coated culture plates from BMMSCs, BMMSCs were seeded and maintained with α-MEM consisting of 15% FBS, (2mM) Glutamine, (55*M*m) 2-Mercaptoethanol, Penicillin/ streptomycin (Life Technologies, NY, USA) and (100μM) L-ascorbic acid (Wako pure, Tokyo, Japan). After 7 days of culturing, the cells were decellularized by incubation with 0.005% Triton X-100 for 5 min at RT. After washing with PBS two times, ECM-coated plates containing PBS were dried 30 min and stored at 37℃ with 5% CO_2 for up to 1 month.

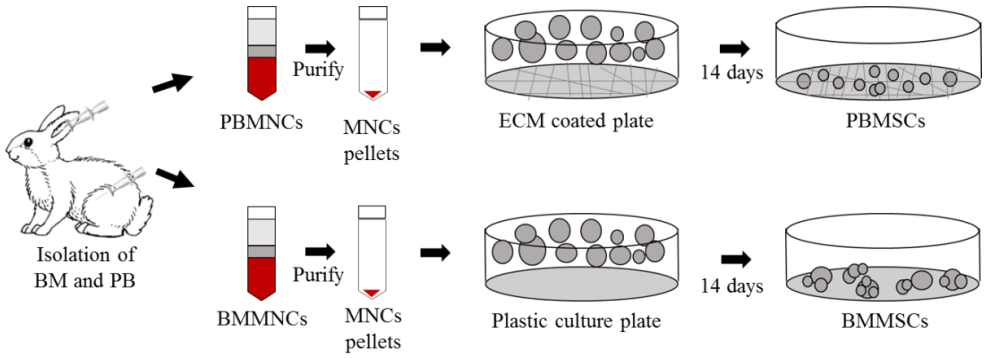


Figure 1. The picture shows the diagram scheme for obtaining PBMSCs. BM, bone marrow; PB, peripheral blood; PBMNCs, peripheral blood mononuclear cells; BMMNCs, bone marrow mononuclear cells; MNCs, mononuclear cells; ECM, extracellular matrix; PBMSCs, peripheral blood mesenchymal stem cells; BMMSCs, bone marrow mesenchymal stem cells.

2. Characterization of the PBMSCs *in vitro* and *in vivo*

2-1. Immunophenotypic identification of MSCs by flow cytometry

Flow cytometry analysis was performed to analyze the surface markers of the PBMSCs. The same number of *in vitro* expanding PBMSCs and BMMSCs were harvested with triple express (Life Technologies, NY, USA), washed with fluorescence-activated cell scanner (FACS) buffer, and thus incubated with 1% blocking solution on ice for 1 hour. They were then incubated with 1 μg of phycoerythrin (PE)-conjugated monoclonal antibodies against anti-CD14 (RMC5-3, Becton-Dkickinson, Bioscience, Germany) for 45 min. Isotype-matched PE-conjugated monoclonal antibody (IgG2a) (Becton-Dkckinson, Bioscience, Germany) was used as a control. The cell suspensions were incubated with 1 μg of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody CD90 (ab226, Abcam, MA, USA) and isotype-matched control IgG1 (15H6, SouthernBiotech, AL, USA) on ice for 1 hour. The cell suspensions were washed and suspended in 1 ml of PBS for analysis using FACS caliber (Becton-Dickinson, Bioscience, CA, USA). For each sample, 10,000 events were used to analysis positive expression of the level of fluorescence. The percentage of positive stained cells was analyzed with the flow cytometry caliber software (Becton-Dickinson, NY, USA).

2-2. *In vitro* cell proliferation assay

Bromodeoxyuridine (BrdU) incorporation assay was conducted to identify cell proliferation ability of the PBMSCs, the same number of *in*

vitro expanding PBMSCs and BMMSCs were seeded on the 8-well chamber slide (Nunc, NY, USA) and cultured in the basic culture medium. After 1 day, the BrdU labeling reagents (Invitrogen, Maryland, USA) were added. The incorporated BrdU was stained with the BrdU staining kit (Invitrogen, CA, USA) after 24 hours. Representative stained images were used to calculate the number of BrdU stained nuclei and the total cells using a microscope.

2-3. *In vitro* cell differentiation potential assay

For osteogenesis differentiation, 70% to 80% both the PBMSCs and the BMMSCs were induced in osteogenic medium which consisted of α-MEM supplemented with 20% FBS (Becton Dickinson, NJ, USA) (2mM) L-glutamine, (55 μM) 2-mercaptoethanol, (Life Technologies, NY, USA), (10nM) dexamethasone (Sigma, NY, USA), (100 μM) L-ascorbic acid (Wako Pure, Tokyo, Japan), (2mM) β-glycerophophate, and penicillin & streptomycin (Life Technologies, NY, USA) for 3 weeks. The induction cells were fixed by 60% isopropanol for 1 min and then stained calcium with Alizarin red S and von kossa solutions for 5 min.

For adipogenesis differentiation, 70% to 80% both the PBMSCs and the BMMSCs were induced in adipogenic medium containing α-MEM supplemented with 20% rabbit serum (life technologies, NY, USA), (2mM) L-glutamine, (55 μM) 2-mercaptoethanol (Life Technologies, NY, USA), penicillin/streptomycin (Life Technologies, NY, USA), (100 μM) L-ascorbic acid (Wako pure, Tokyo, Japan), (0.5mM) isobutyl-methylxanthine (Sigma, NY, USA), (60 μM) indomethacin (Sigma, NY, USA), (0.5 μM) hydrocortisone (Sigma, NY, USA), (10 μM) insulin

(Sigma, Saint Louis, USA). The inducing differentiation cells were fixed with 10% formalin for 10 min at RT and demonstrated by the accumulation of lipid droplet which stained with Oil Red O solutions for 30 min.

For chondrogenesis differentiation, 70% to 80% both the PBMSCs and the BMMSCs were cultured in chondrogenic medium containing α-MEM supplemented with 15% FBS (Becton Dickinson, NJ, USA), (2mM) L-glutamine (Life Technologies, NY, USA), (100μM) L-ascorbic acid (Wake pure, Tokyo, Japan), (10nM) dexamethasone (Sigma, NY, USA), 1 ng/ml TGF-b (Sigma, NY, USA), 50 mg/ml insulin transferin selenium serum acid (Sigma, NY, USA). Four weeks after induction, the cell pellets were harvested, fixed overnight with 4% paraformaldehyde (PFA), dehydrated in ascending concentrations (70%, 80%, 90%, 95%, and 100%) of the ethanol, and embedded in paraffin wax. The sections were then stained by Alcian blue and Safranin-O respectively at RT.

2-4. *In vivo* bone-forming transplantation assay

To further characterize the osteogenic potential ability of the PBMSCs *in vivo*, PBMSCs were suspended in 1ml of α-MEM and mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) powder (Zimmer, Freiburg, Germany). Previous experiments showed that a porous ceramic consisting of hydroxyapatite (HA) and tricalcium/ phosphate (TCP) at a 60/40 ratio exhibited significant bone formation after subcutaneous transplantation[19]. The same number of *in vitro* expanding BMMSCs were used as control. The transplants were placed in subcutaneous pockets of the immunocompromised mice (BALB/c).

After ten weeks, the pellets were got out, fixed with 4% formalin, decalcified with buffered 10% eidetic acid, and then embedded in paraffin. Sections were deparaffinized and stained with haematoxylin and eosin (H&E). This surgical procedure was performed in accordance with specifications of an approved small animal protocol (NIDCR #00-113).

3. Bone regeneration capacity of the PBMSCs in double-canaled implant

3-1. Double-canaled implants preparation and injection of PBMSCs-HA/TCP, BMMSCs-HA/TCP, and HA/TCP

A total of 80 screw-shaped with double-canaled implants made of grade 4 commercially pure Titanium (Ti) were prepared as previously study described[17]. The total length of the implant was 7 mm with 5 mm threaded and 2 mm unthreaded at the coronal portion. The implant had an outer diameter of 3.75 mm, a thread pitch height of 0.6 mm, two canals of 1.5 mm in diameter passing through the thread part, and 1.0 mm apart from each canal which was designed according to the position of bone quality (upper canal to cortical area and lower canal to marrow area) (Fig. 2A). The internal surfaces of the canal were machined. The same number of *in vitro* expanding PBMSCs and BMMSCs were suspended in 1ml of α-MEM and mixed with 10 mg of HA/TCP (Zimmer, Freiburg, Germany) powder graft materials with a pore size of 300 - 500 μm and incubated in a humidified atmosphere at 37℃ with 5% CO_2 for 2 hrs. Defect only and filling the canals with HA/TCP, and filling the canals with PBMSCs and BMMSCs mixing with HA/TCP were prepared to examine

the capacity of the PBMSCs to bone regeneration in the cortical and marrow areas of tibia. Four different groups were prepared as the follows; Defect only group: canals were not filled (Fig. 2B-a), HA/TCP group: canals filled with HA/TCP (Fig. 2B-b), BMMSCs group: canals filled with BMMSCs mixing with HA/TCP (Fig. 2B-c), PBMSCs group: canals filled with PBMSCs mixing with HA/TCP (Fig. 2B-d).

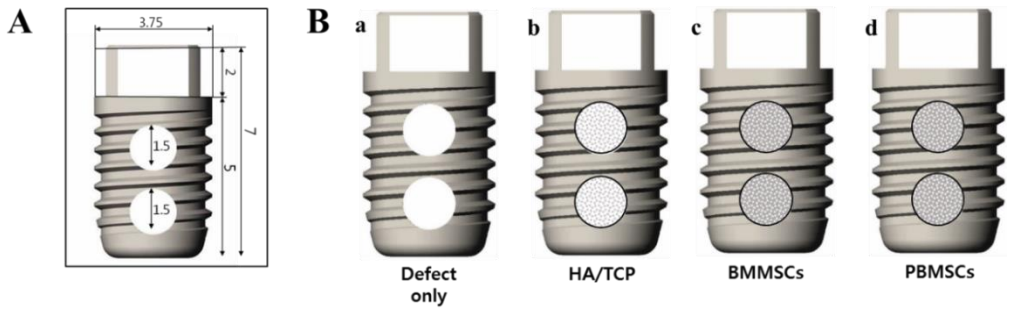


Figure 2. Preparation of the double-canaled implant. (A) The image of threaded titanium double-canaled implant. The total length of the implant was 7 mm with 5 mm threaded and 2 mm unthreaded at the coronal portion. The implant had an outer diameter of 3.75 mm, two canals of 1.5 mm in diameter. **(B)** The upper and lower implant canals were prepared: (a) Defect only group; canals were not filled, (b) HA/TCP group; canals filled with HA/TCP, (c) BMMSCs group; canals filled with BMMSCs mixing with HA/TCP, and (d) PBMSCs group; canals filled with PBMSCs mixing with HA/TCP. HA/TCP, hydroxyapatite/tricalcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells.

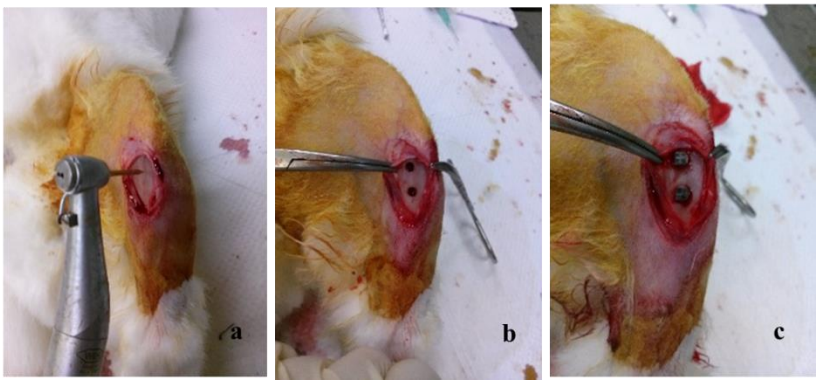
3-2. surgical procedure and implant installation

All rabbits were performed under general anesthesia with intramuscular injection of ketamine (Yuhan, Gunpo, Korea) and 0.15 mg/kg Rompun (Bayer, Ansan, Korea) and local anesthesia with 1.0 ml of 2% lidocaine including 1:100,000 epinephrine (Yu-han, Gunpo, Korea) at the tibia under aseptic condition. Prior to surgery, the animal tibia of operating sites were shaved, washed, and decontaminated with iodine. After surgical draping, incisions were made to expose the tibia, blunt dissection of the muscles, and elevation of the periosteum were performed. The implant bed was prepared using a pilot drill (1.2 mm), followed by a step drilling (3.3 mm) and tapping. The holes were drilled with a low speed rotary instrument under constant irrigation with sterile saline. A total of four implants were randomly placed in the right and left tibias (Fig. 3A). The implants were inserted in a depth to allow penetrating the first cortical bone area only with a torque of 30Ncm. The upper canal was situated in the cortical bone area of tibia (Fig. 3B-a), and the lower canal was located in the marrow space area (Fig. 3B-b). The surgical sites were closed in layers with the muscle, fascia. Internal dermal layers were sutured with 4-0 Vicryl (Vicryl Plus, Ethicon, USA) and the outer dermis was sutured with 3-0 chromic gut (Vicryl Plus, Ethicon, USA). The animals were monitored during recovery for any possible complications, given water and chow during the healing period. At 3 weeks and 6 weeks after implant placement, the animals were euthanized. The implants were surgical exposed by sharp dissection of the bone, then removed en bloc with surrounding bone, and stored in 10% formalin.

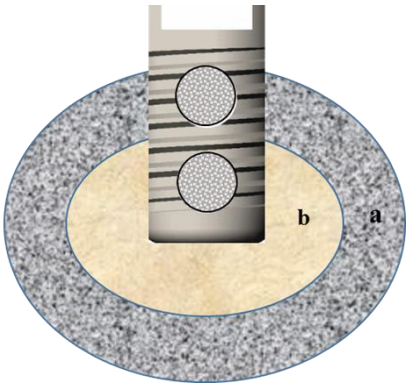
3-3. Specimen preparation, histological and histomorphometric analysis

Ten rabbits were sacrificed after 3 weeks and 6 week for histological and histomorphometric analysis, respectively. All 80 implants with surrounding bone were removed en bloc, fixed in neutral buffered formalin, dehydrated in 70%, 80%, 90%, 95%, and 100% alcohol, and embedded in a light curing resin (Technovit 7200 VLC, Wehrheim, Germany). The embedded implants were divided longitudinally by the sewing machine (Exakt, Norderstedt, Germany). The sections were ground to approximately 40 μm thick, and stained with 1% toluidine blue and von kossa for collagen staining. The central section was used for histological and histomorphometric analysis. The percentages of new bone (NB) inside the double-canaled were defined as the area of newly formed bone inside the double-canaled divided by the complete area of the double-canaled. The percentages of graft material (GM) inside the double-canaled were defined as the area of the graft particles divided by the complete area of the double-canaled. The percentages of marrow space (MS) inside the double-canaled were defined as the area of the rest spaces except for NB and GM areas (Fig 3C).

A



B



C

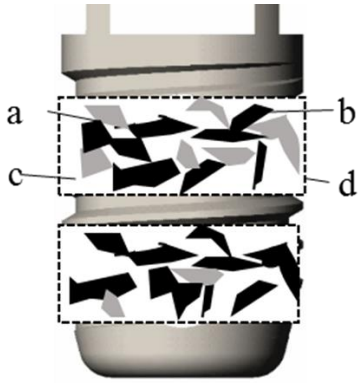


Figure 3. Surgical procedure and schematic diagram of the histomorphometric analysis. (A) The surgical procedure of the double-canaled implant placement. (a) Incisions were made to expose the tibia, and the implant bed was prepared using a pilot drill. (b) The double-canaled implants were inserted in a depth to allow penetrating the first cortical bone area. (c) A total of four implants were randomly placed in the right and left tibias. **(B)** Cross-sectional diagram of the double-canaled implant installation into rabbit tibia. The upper canal was positioned in the cortical bone area (a) and the lower canal was positioned in the marrow space area (b). **(C)** Schematic diagram of the histomorphometric analysis. (a) = newly formed bone areas in the double-canaled implant. (b) = graft material areas in the double-canaled implant, (c) = marrow spaces in the double-canaled implant, and (d) = total area of the double-canaled implant.

4. Statistics

The data were expressed as mean±standard deviation. The characterization study *in vitro* and bone regeneration study in immunocompromised mice data were analyzed with Student's *t* test. A multivariate analysis of variance was utilized to compare the results of histomorphometric measurements after implantation, and *post hoc* analysis was dong with the Tukey test. *P* values less than 0.05 were considered to be significant in all tests.

Ⅲ. RESULTS

1. Characterization of the PBMSCs *in vitro*

1-1. Morphology, proliferation and Immunophenotypic analysis of the PBMSCs

The MNCs from PB were purified by red cell blood lysis and PBMSCs were obtained according to their adherence to ECM-coated plate. After 3 to 4 days of primary seeding, the PBMNCs were bound to the ECM-coated plate. After 14 days of primary seeding, the PBMSCs (Fig. 4A-b) showed a typical homogeneous fibroblast-like shape as BMMSCs (Fig. 4A-a), and grew into colony when the cells were sub-cultured on the plastic culture plates. There was no statistically significant difference in proliferation rate between BMMSCs (Fig. 4B-a) (82.79% ± 0.09%) and PBMSCs (Fig. 1B-b) (87.10% ± 0.06%) when these cells were sub-cultured on plastic culture plate (Fig. 4B-c). Immunophenotypic analysis by flow cytometry indicated that BMMSCs were negatively expressed for CD14 (Fig. 4C-a) (3.67% ± 0.31%) and positively expressed for CD90 (Fig. 4C-c) (47.09% ± 0.58%), while PBMSCs were negatively expressed for the hematopoietic stem cells marker CD14 (Fig. 4C-b) (0.85% ± 0.01%) and positively expressed for the MSC marker CD90 (Fig. 4C-d) (31.79% ± 1.72%). However, there expression was statistically difference between the BMMSCs and the PBMSCs (Fig. 4C-e) ($p < 0.05$).

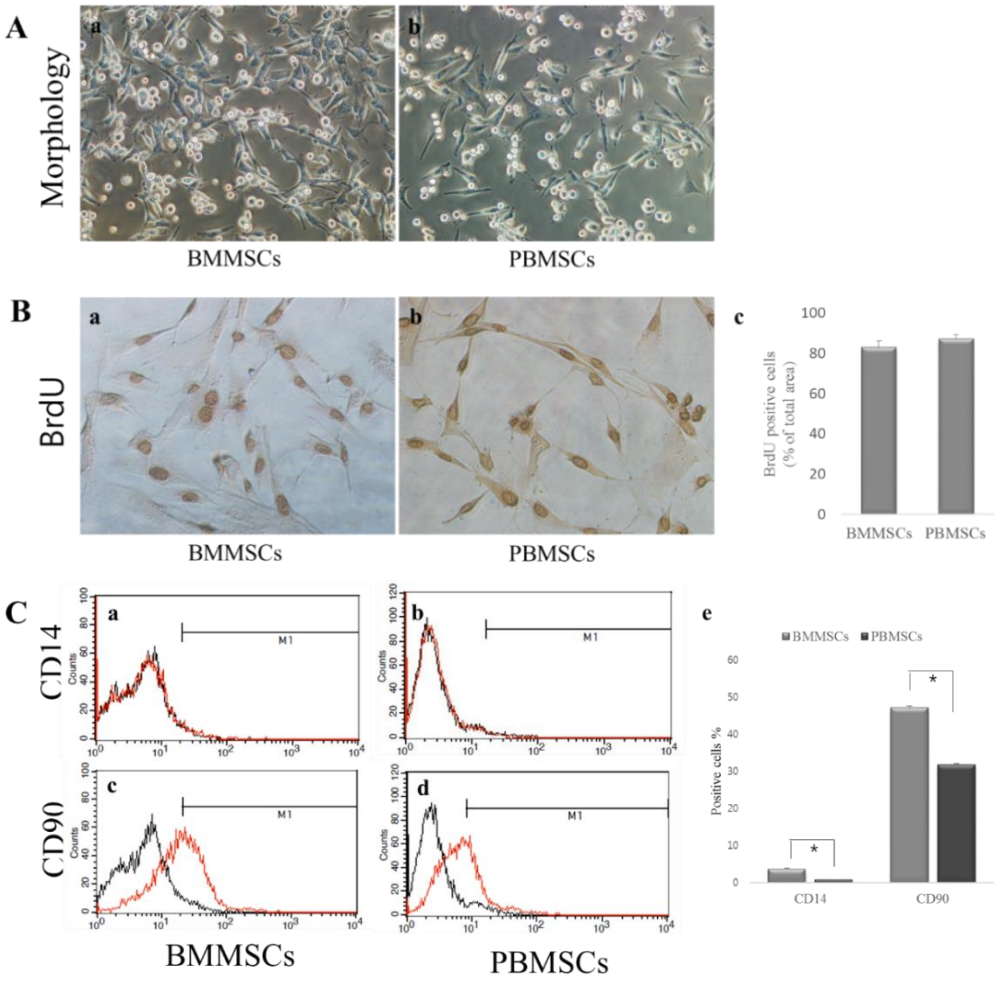

A
Morphology
a
b
BMMSCs
PBMSCs
B
BrdU
a
b
BMMSCs
PBMSCs
c
BrdU positive cells (% of total area)
100
80
60
40
20
0
BMMSCs
PBMSCs
C
CD14
CD90
a
b
c
d
M1
Counts
BMMSCs
PBMSCs
e
BMMSCs
PBMSCs
Positive cells %
60
50
40
30
20
10
0
*
*
CD14
CD90

Figure 4. Characterization of the PBMSCs. (A) The morphological character of the BMMSCs (a) and the PBMSCs (b) under inverted phase contrast microscope, X 200. Both the PBMSCs and BMMSCs displayed a typical fibroblast-like shape. **(B)** The proliferation rate of the BMMSCs (a) and the PBMSCs (b) cultured on a plastic culture plate, X 400. Incorporated BrdU were stained in the BMMSCs and the PBMSCs. (c) There was no significantly difference percentage of BrdU positive nuclei cells relative to the total nucleated cells between BMMSCs and PBMSCs. **(C)** Immunophenotypic identification of the BMMSCs and the PBMSCs by flow cytometry. Flow cytometry analysis of the cultured BMMSCs and PBMSCs, BMMSCs and PBMSCs negatively expressed for HSCs marker CD14 (a, b), but these cells positively expressed for the MSCs marker CD90 (c, d). Black histogram represent isotype-matched antibody control staining. (e) The analysis revealed that the BMMSCs showed higher expression of MSC and HSC surface marker than the PBMSCs, $*p < 0.05$, the graph bar shows mean±SD. BMMSCs, Bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells; BrdU, bromodeoxyuridine; CD14, cluster of differentiation 14; CD90, cluster of differentiation 90.

1-2. Differentiation potential of the PBMSCs in *vitro* assay

For osteogenesis potential, Both the PBMSCs and the BMMSCs morphology were changed from a spindle shape into a more polygonal appearance after induction into osteogenesis medium. The formed nodules were stained with red by Alizarin red S (Fig 5A-a, b) and changed to black by staining with von kossa (Fig. 5A-c, d). The PBMSCs (69.07% ± 7.53%) were able to form mineralized nodules as BMMSCs (72.76% ± 1.56%) (Fig 5E). For adipogenesis potential, the PBMSCs had capacity to differentiate into lipid droplets which were appeared red by Oil Red O staining (Fig. 5B). The adipogenic differentiation capacity of

the PBMSCs (57.65% ± 3.07%) was similar to the BMMSCs (57.93% ± 1.26%) (Fig. 5F). The PBMSCs were capable of forming cartilage matrix, as seen in BMMSCs (Fig. 5C).

2. Characterization of the PBMSCs *in vivo*

Differentiation potential of the PBMSCs *in vivo* transplantation assay

An *in vivo* transplantation bone-forming assay which PBMSCs and BMMSCs were implanted subcutaneously into immunocompromised mice was used to obtain the further evidence of the osteogenic potential ability of the PBMSCs. Histological examination of the implanted PBMSCs (Fig. 5D-b) showed clear evidence of the mineralized bone formation as BMMSCs (Fig. 5D-a). The amount of bone formation between PBMSCs (40.94% ± 3.87%) and BMMSCs (43.01 ± 3.97) were not significantly difference (Fig. 5G).

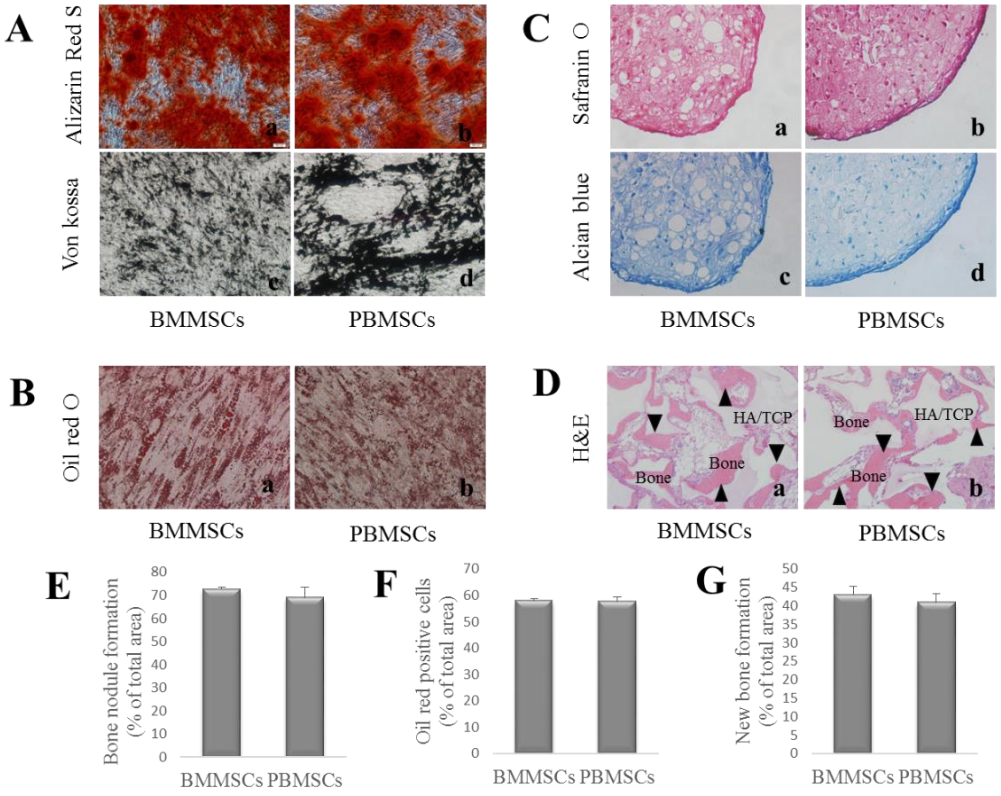

A
Alizarin Red S
Von kossa
a
b
c
d
BMMSCs
PBMSCs
C
Safranin O
Alcian blue
a
b
c
d
BMMSCs
PBMSCs
B
Oil red O
a
b
BMMSCs
PBMSCs
D
H&E
HA/TCP
Bone
Bone
a
Bone
HA/TCP
Bone
b
BMMSCs
PBMSCs
E
Bone nodule formation (% of total area)
80
70
60
50
40
30
20
10
0
BMMSCs PBMSCs
F
Oil red positive cells (% of total area)
70
60
50
40
30
20
10
0
BMMSCs PBMSCs
G
New bone formation (% of total area)
50
45
40
35
30
25
20
15
10
5
0
BMMSCs PBMSCs

Figure 5. Differentiation potential of the PBMSCs. (A) Osteocytes were detected by the deposition of calcium mineralization nodules staining with Alizarin red S (a, b) and von-kossa (c, d). **(B)** Adipocytes was indicated by formed small lipid droplets and turned red upon Oil red O staining, BMMSCs (a), PBMSCs (b). **(C)** Chondrocytes were assessed by Safranin-O (a, b) and Alcian blue (c, d) staining for acidic sulfate mucosubstances. Histological sections of the pellets were stained by Alcian blue and Safranin-O for observation of cartilage matrix. **(D)** Evidence of osteogenic potential of the BMMSCs and the PBMSCs implanted into immunocompromised mouse. After 10 weeks of transplantation, mineralized bone was formed on the surface of the HA/TCP in both the BMMSCs (a) and the PBMSCS (b). There were no significant difference osteogenesis **(E)**, adipogenesis **(F)** and *in vivo* osteogenic potential **(G)** differentiation potential between the BMMSCs and the PBMSCs. (The graph bar represents mean ± SD) BMMSCs, Bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells; HA/TCP, hydroxyapatite/tricalcium phosphate; H&E, haematoxylin and eosin.

3. Bone regeneration capacity of the PBMSCs in double-canaled implant

Histological and histomorphometric analysis

All animals recovered well from the surgical intervention without any significant complications and were sacrificed according to the schedule. None of the specimens showed evidence of severe inflammation of foreign body reaction. In all sections, the upper canals were situated in the cortical bone area and the lower canals were placed in the marrow space area at 3 weeks and 6 weeks (Fig. 6A, Fig. 6B, Fig. 7A, Fig. 7B, Fig. 8A, Fig. 8B, Fig. 9A, and Fig. 9B).

At 3 weeks, the upper canals of the defect-only group (Fig. 6B-a, Fig. 7B-a) showed newly formed bone which mainly growing into the canal from the outside. At higher magnification (Fig. 6C-a, Fig. 7C-a), the newly formed bone was located mainly in the lateral sides of the upper canals. In the lower canals of the defect-only group (Fig. 6B-e, Fig. 6C-e, Fig. 7B-e, Fig. 7C-e), mostly fatty marrow spaces were observed. In the upper canals of the HA/TCP group (Fig. 6B-b, Fig.7B-b), the remnants of the HA/TCP scaffold and a smaller amount of mineralized bone tissues were observed. At higher magnification (Fig. 6C-b, Fig. 7C-b), the mineralized bone tissues, fibrous tissue encapsulation appear around the HA/TCP, and a few mature bone were observed in the canal from outside the canal. In the lower canals of the HA/TCP group (Fig. 6B-f, Fig. 7B-f), Mostly remnants of the HA/TCP scaffold and the fatty marrow spaces were observed, while fibrovascular tissues less invaded HA/TCP at higher magnification (Fig. 6C-f, Fig. 7C-f). In the upper canals of the BMMSCs group (Fig. 6B-c, Fig. 6C-c, Fig. 7B-c, Fig. 7C-c) and the PBMSCs (Fig. 6B-d, Fig. 6C-d, Fig. 7B-d, Fig 7C-d) group, a little of mature mineralized woven bone with osteogenic marker positive cells was detected in the central part of the canals. In the lower canals of the BMMSCs group (Fig. 6B-g, Fig. 6C-g, Fig. 7B-g, Fig. 7C-g) and the PBMMSCs (Fig. 6B-h, Fig. 6C-h, Fig. 7B-h, Fig. 7C-h) group, fibrovascular tissues with osteogenic marker positive cells was mainly detected. A few of mineralized woven bone was detected.

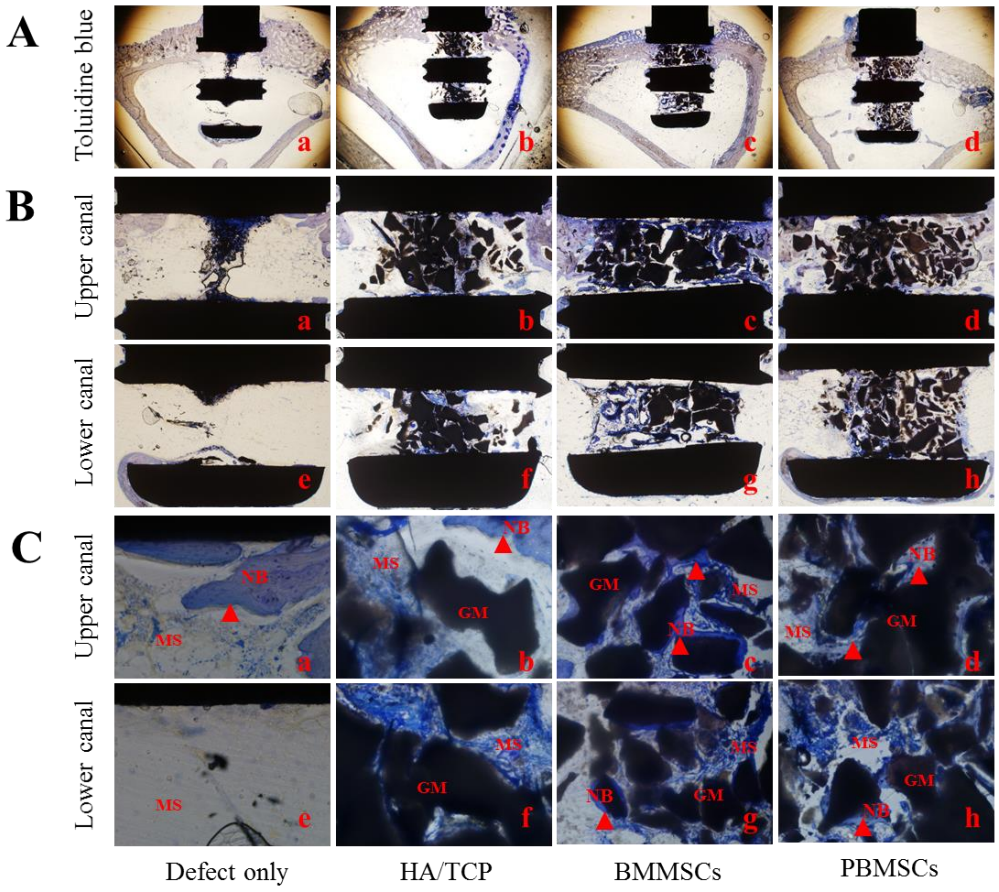


Figure 6. Histological analysis of the threaded titanium double-canaled implant for toluidine blue staining at 3 weeks. (A) X 12.5. (a) Defect only, (b) HA/TCP, (c) BMMSCs mixing with HA/TCP, (b) PBMSCs mixing with HA/TCP. **(B)** X 40. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal; **(C)** X 200. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal. New bone (NB), graft material (GM), and marrow space (MS) were observed. HA/TCP, hydroxyapatite/tricalcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells.

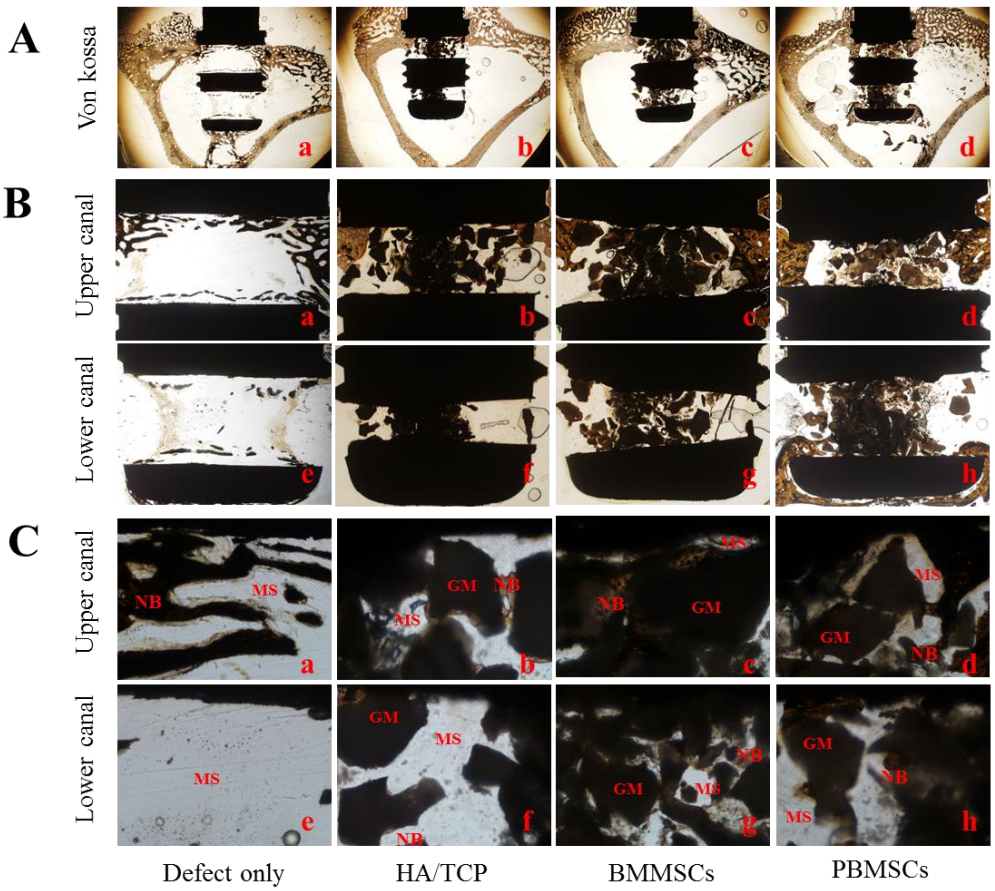


Figure 7. Histological analysis of the threaded titanium double-canaled implant for von kossa staining at 3 weeks. (A) X 12.5. (a) Defect only, (b) HA/TCP, (c) BMMSCs mixing with HA/TCP, (b) PBMSCs mixing with HA/TCP. **(B)** X 40. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal; **(C)** X 200. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal. New bone (NB), graft material (GM), and marrow space (MS) were observed. HA/TCP, hydroxyapatite/tricalcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells.

At 6 weeks, the upper canals of the defect-only group (Fig. 8B-a. Fig. 8C-a, Fig. 9B-a, Fig. 9C-a), more newly formed bone showed a configuration extending toward the central part of the canal from the lateral side. Most of the fatty marrow spaces were observed in the lower canals of the defect-only group (Fig. 8B-e, 8C-e, Fig. 9B-e, Fig. 9C-e). In the upper canals of the HA/TCP group (Fig. 8B-b, Fig. 9B-b), mature mineralized woven bone with osteogenic marker positive cells was appeared around the HA/TCP. At higher magnification (Fig. 8C-b, Fig. 9C-b), few gaps were presented at the bone particle interface. In the lower canals of the HA/TCP group (Fig. 8B-f, Fig. 8C-f, Fig. 9B-f, Fig. 9C-f), a small amount of newly formed woven bone with few invasion of the fibrovascular tissues were observed. In the upper canals of the BMMSCs group (Fig. 8B-c, Fig. 9B-c) and the PBMMSCs (Fig. 8B-d, Fig. 9B-d) group, mature bone could be detected in the central part of the canals. At higher magnification (Fig. 8C-c, Fig. 8C-d, Fig. 9C-c, Fig. 9C-d), no gaps were presented at the surface of the particles in contact with newly formed bone. The mineralized matrix of the newly formed bone consists of mature lamellar-like bone together with numerous rounded osteocytes which were very well-organized. There was no fibrovascular tissue detected around the HA/TCP. In the lower canals of the BMMSCs group (Fig. 8B-g, Fig. 9B-g) and the PBMSCs (Fig. 8B-h, Fig. 9B-h) group, there was also few quantities of mature bone, moreover, bone with osteogenic marker positive cells could be seen at high magnification (Fig. 8C-g, Fig. 8C-h, Fig. 9C-g, Fig. 9C-h).

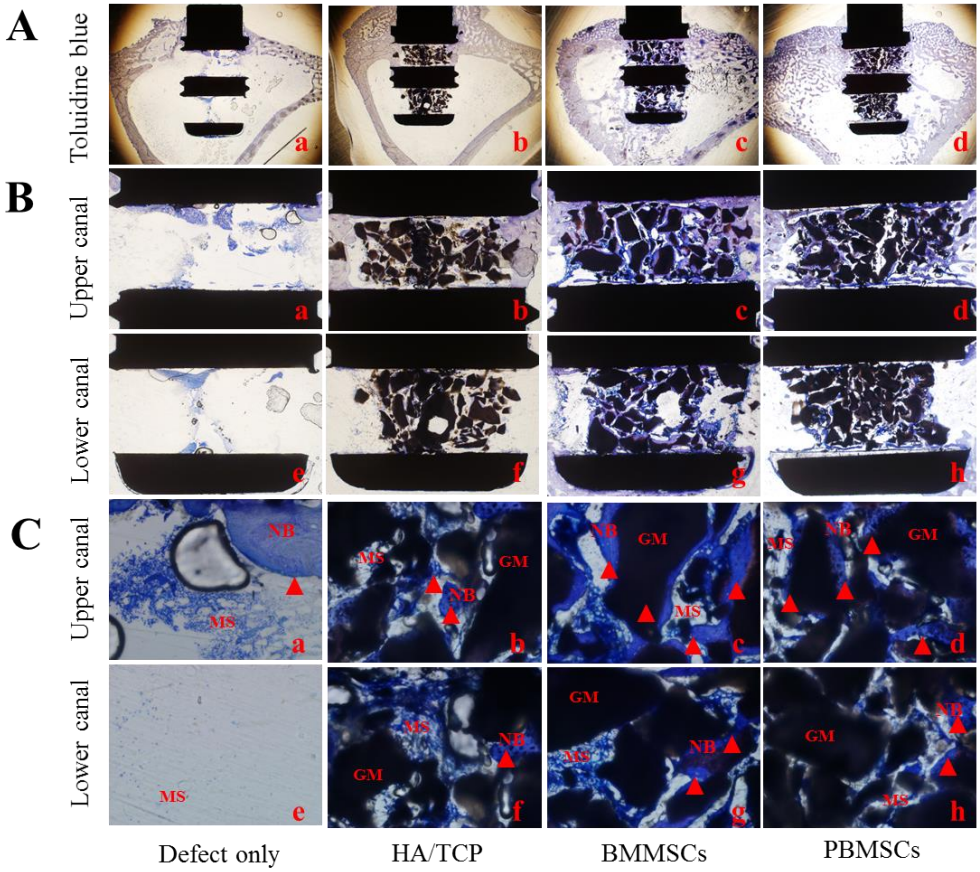


Figure 8. Histological analysis of the threaded titanium double-canaled implant for toluidine blue staining at 6 weeks. (A) X 12.5. (a) Defect only, (b) HA/TCP, (c) BMMSCs mixing with HA/TCP, (b) PBMSCs mixing with HA/TCP. **(B)** X 40. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal; **(C)** X 200. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal. New bone (NB), graft material (GM), and marrow space (MS) were observed. HA/TCP, hydroxyapatite/tricalcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells.

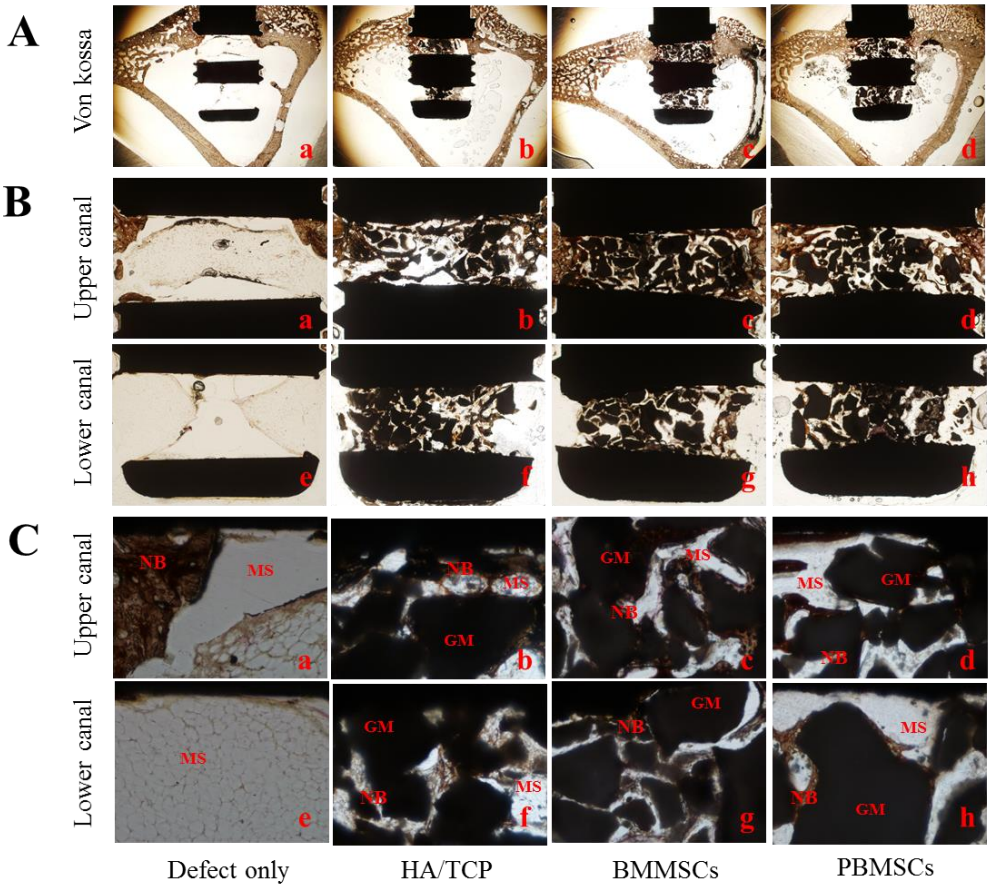
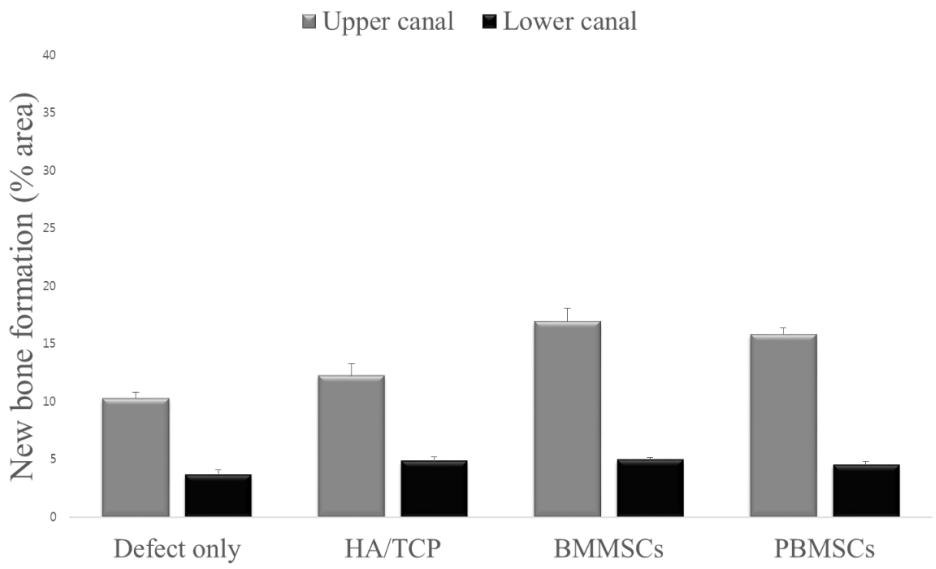


Figure 9. Histological analysis of the threaded titanium double-canaled implant for von kossa staining at 6 weeks. (A) X 12.5. (a) Defect only, (b) HA/TCP, (c) BMMSCs mixing with HA/TCP, (b) PBMSCs mixing with HA/TCP. **(B)** X 40. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal; **(C)** X 200. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixing with HA/TCP, (d, h) PBMSCs mixing with HA/TCP; Rows top to bottom: upper canal and lower canal. New bone (NB), graft material (GM), and marrow space (MS) were observed. HA/TCP: hydroxyapatite/tricalcium phosphate, BMMSCs: bone marrow mesenchymal stem cells, PBMSCs: peripheral blood mesenchymal stem cells.

There was no significant difference bone regeneration capacity between the BMMSCs group and the PBMSCs group in the upper and lower canals. Inside the upper canals at 3 weeks, the BMMSCs group (16.92% ± 3.58%) and the PBMSCs (15.81% ±1.86%) group showed more NB than the HA/TCP group (12.22% ± 3.40%) and the defect only group (10.28% ± 1.71%)($p < 0.05$). However, there was no difference NB among the defect only group (3.65% ±1.34%), the HA/TCP group (4.82% ± 1.15%), the BMMSCs group (4.92% ± 1.15%), and the PBMSCs group (4.51% ± 0.89%) in the lower canals at 3 weeks ($p > 0.05$) (Fig. 10A). Inside the upper and lower canals, the BMMSCs group (upper canal: 35.45% ± 5.74%, lower canal: 7.26% ± 1.10%) and the PBMSCs group (upper canal: 36.30% ± 3.71%, lower canal: 7.56% ± 1.20%) showed significantly more NB than the HA/TCP group (upper canal: 21.48% ± 4.87%, lower canal: 5.86% ± 1.03%) and the defect only group (upper canal: 14.69% ± 2.61%, lower canal: 4.94% ± 0.77%) at 6 weeks ($p < 0.05$). However, there was no significant difference NB between the BMMSCs group and the PBMSCs group, followed by the HA/TCP group and the defect only group ($p > 0.05$) (Fig. 10B). At 3 weeks and 6 weeks inside the upper canals, the HA/TCP group showed more NB than the defect only group ($p < 0.05$). The detailed percentages of NB, GM, and MS at 3 weeks and 6 weeks were described in the table1 and table 2.

A


Upper canal
Lower canal
New bone formation (% area)
40
35
30
25
20
15
10
5
0
Defect only
HA/TCP
BMMSCs
PBMSCs

B

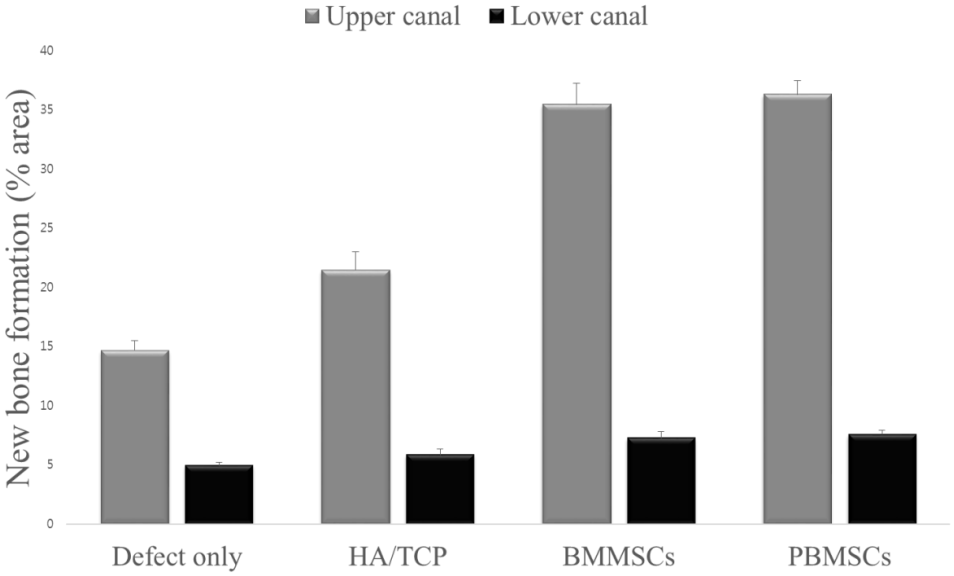

Upper canal
Lower canal
New bone formation (% area)
40
35
30
25
20
15
10
5
0
Defect only
HA/TCP
BMMSCs
PBMSCs

Figure 10. Histomorphometric analysis (A) New bone formation inside the upper and lower implant canals implanted into the rabbit for 3 weeks. **(B)** New bone formation inside the upper and lower implant canals implanted into the rabbit for 6 weeks. (the graph bar represents means ± SD). NB, new bone; GM, graft material; MS, marrow space; HA/TCP, hydroxyapatite/tricalcium phosphate; BMMSCs, bone marrow mesenchymal stem cells; PBMSCs, peripheral blood mesenchymal stem cells.

Table 1. Histomorphometric values in the canals at 3 weeks

	Groups	NB (%)	GM (%)	MS (%)
Upper canal	Defect only	10.28 ± 1.71	-	89.72 ± 1.71
	HA/TCP	12.22 ± 3.40	41.21 ± 3.60	46.57 ± 5.51
	BMMSC mixed with HA/TCP	16.92 ± 3.58	40.57 ± 5.53	42.50 ± 4.73
	PBMSC mixed with HA/TCP	15.81 ± 1.86	41.33 ± 4.13	42.87 ± 4.40
Lower canal	Defect only	3.65 ± 1.34	-	96.35 ±1.34
	HA/TCP	4.82 ± 1.15	39.39 ± 2.67	55.79 ± 2.90
	BMMSC mixed with HA/TCP	4.92 ± 0.78	37.87 ± 2.33	57.21 ± 2.76
	PBMSC mixed with HA/TCP	4.51 ± 0.89	39.52 ± 2.25	55.97 ± 2.87

Table 2. Histomorphometric values in the canals at 6 weeks

	Groups	NB (%)	GM (%)	MS (%)
Upper canal	Defect only	14.69 ± 2.61	-	85.31 ± 2.61
	HA/TCP	21.48 ± 4.87	40.36 ± 2.96	38.16 ± 5.69
	BMMSC mixed with HA/TCP	35.45 ± 5.74	40.41 ±1.94	24.14 ± 6.16
	PBMSC mixed with HA/TCP	36.30 ± 3.71	40.68 ± 2.08	23.03 ± 3.49
Lower canal	Defect only	4.94 ± 0.77	-	95.06 ± 0.77
	HA/TCP	5.86 ± 1.03	38.98 ± 1.78	55.17 ± 2.27
	BMMSC mixed with HA/TCP	7.26 ± 1.10	40.07 ± 1.97	52.68 ± 2.20
	PBMSC mixed with HA/TCP	7.56 ± 1.20	39.60 ± 2.14	52.84 ± 2.32

Ⅳ. DISCUSSION

PB have several advantages in basic researches and clinical applications over BM. PB are easier and safer to be collected than BM which must be extracted from bone. Isolation of MSCs from PB have important clinical implication for cell-based bone regeneration. Contrasting to BM or other sources of MSCs, the isolation of PB is minimally invasive, and does not lead further complications to the donor site. At the same time, it is unclear and controversial about the method of isolation and purification of MSCs from PB. At present, the most common method of isolating MSCs from PB is density gradient centrifugation and then specific progenitor cells can be purified after isolating. Previously study have been isolated and characterized the PBMSCs using the different isolation methods, such as selecting the MSCs via simple replacement of the medium after the first cell seeding[20], and using the fibrin matric to obtain the MSCs[15], however, the results are dissatisfied. In this study, we endeavored to acquire MSCs from PB of rabbit through the method of culturing PBMNCs on ECM-coated culture plate. Our

results demonstrated that the rabbit PB fibroblast-like MSCs have similar phenotypic, proliferation and differentiation characteristics contrasting to BMMSCs which were prepared from the BM of rabbit. MSCs isolated from PB showed a typical spindle fibroblast-like morphology in their adherence to plastic culture plate. The ECM-coated culture plate was effective of obtaining the PBMSCs. Our previous study have been reported that the ECM-coated plates were effective of obtaining the suspended cells which were isolated from BM[17]. Similar results were also observed by other author, in which showed the ECM facilitated the expansion of BMMSCs[21]. While other previous researches have been suggested that the ECM was a significant component of collagen types I and III, fibronectin, and a major component of basement membrane[16]. The ECM favors the proliferation, self-renewal, and multi-potential differentiation, which suggests that ECM secreted by BMMSCs modulates behavior of cells and facilitates retention of MSCs properties[22]. Therefore, further study should clearly state what component of ECM control the MSCs binding mechanism in the future. There was evidence recently that MSCs existed in the suspended cell populations which isolated from mouse BM[23]. Additionally, we recently reported that ECM could bind the suspended cells which isolated from rabbit BM and was valuable for propagation of the suspended cells[17]. It was plausible that MSCs detected from PB were relation to the suspended cell populations from BM. There may be some specific cytokines and factors contained in the ECM which could control the correlation between the suspended cell and the PBMSCs. Therefore, further study should clearly state characterizations of both the suspended cells and the PBMSCs, also the possible detailed mechanisms of cytokines and factors included in the

ECM still needed to state. The method of culturing on ECM was valuable for acquiring the MSCs from PB for cell research.

The animal model of this study was rabbit, whereas standards of MSC surface markers established by the international society for cellular therapy were applied to human MSCs only[24]. There are still no consistent standards of MSCs markers for rabbit models. We have done our best to find markers in order to identify PBMSCs. Thus we selected CD14 as a negative and CD90 as a positive marker in this study. Also PBMSCs can differentiate into osteocytes, adipocytes, and chondrocytes under appropriate conditions *in vitro* as BMMSCs. In addition, after 10 weeks of transplantation, new bone and many blood vessels were seen in groups of cells loading HA/TCP, indicating that the PBMMSCs have the osteogenic potential without rejecting reaction, immunological reaction and toxicity, which is similar to BMMSCs *in vivo.* Hence, the characteristics of the MSCs from PB were differ from hematopoietic stem cells, and the MSCs isolated from PB were identified have similar biological characteristics *in vitro* and *in vivo* as BMMSCs. However, the difference species between rabbit and human have also to be considered. PBMSCs were found with ease in lower species, such as mouse, rat, and guinea pig[2]. More basic research work is needed to address these applications, before PBMSCs can be used as a reliable source in human model.

In this study, we utilized the double-canaled dental implant for useful applications of cells and quantifications of bone regeneration in the cortical and marrow areas of the rabbit tibia. The double-canaled implant was the easiest way to prepare and detect bone regeneration according to the bone quality[18]. In spite of the fact that bone regeneration capacity

usually increase from 3 to 6 weeks of observation, the present study was not an experiment over time and it would be possible that individual effect of rabbit can exceed the time factor in animal[25]. In histologic analysis, our previous study reported that bone formation in rabbit was noticeably higher in cortical bone than in marrow space area at 4 weeks, while the same results were observed in this study at 3 weeks and 6 weeks. It has been reported that bone growth was more pronounced in the cortical bone area than the marrow space area in rabbit tibia model[26]. In the same double-canaled implant model, merely 17% and 5% NB area of the HA/TCP group, 28% and 10% NB area of the BMMSCs group were reported in the upper and lower canals, respectively[17]. In the current study, the regenerated NB area in the HA/TCP group were each 12% and 4.8% in the upper and lower canals at 3 weeks. Anyway, the regenerated NB area in the BMMSCs group and the PBMSCs group were merely 17% and 15% in the upper canals, 4.9% and 4.5% in the lower canals at 3 weeks. Moreover, the regenerated NB area in the BMMSCs group and the PBMSCs group were merely 35% and 36% in the upper canals, 7.2% and 7.5% in the lower canal at 6 weeks. It can be estimated that BMMSCs and PBMSCS were on the initial stage of osteogenic differentiation potential in this study at 3 weeks. Previous study has been reported that woven mineralized bone was generally characterized by orientation of collagen fibrils with great number of osteocytes[27]. The similar observation described that early oriented remodeling phase of the woven bone was noted in the osseointegration processes after two week[28], this observation can be found in histological results of this paper.

There may be some limitations on present study, for example, how long transplanted MSCs could be survived, and what the definite role of

transplanted MSCs playing in the bone regeneration. However, the results of this study imply that both the MSCs might have had a participating role in the regeneration of the bone. The ratio between the cortical and the marrow area of the rabbit tibia and mechanical stability of the canals seem to be affected easily because of the difference of bone areas. The compact holding bone provided a firm mechanical interlocking of the canal, which was important for the bone regeneration. Moreover, the different biologic environments would affect the MSCs differentiation potential[29]. With the limitations of this study, we demonstrated that the transplantation of BMSMCs and PBMSCs mixing with HA/TCP groups were more effective than HA/TCP or defect group for bone regeneration. The PBMSCs acquired by the method of culturing on the ECM are multipotent *in vitro* and *in vivo* in this study, indicating prospects of broad applications in the field of the cell-based bone regeneration in the future.

Ⅴ. CONCLUSIONS

All in all, MSCs were successfully acquisited from PB using the method of culturing on the ECM-coated culture plate. PBMSCs have the similar biological characteristics *in vitro* and *in vivo* as BMMSCs, including morphology, proliferation and differentiation capacity. MSCs derived from PB showed the same ability to bone regeneration just like BMMSCs. The bone formation area percentages were significantly greater in the cells groups than the HA/TCP or the defect groups, both in the cortical area at 3 weeks and 6 weeks and in the marrow area at 6 weeks. Still there was no significant difference among all groups in the marrow area at 3 weeks. Consequently, the PBMSCs could be one of the most promising source for bone regeneration in cell therapy.

REFERENCES

[1] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418:41-9.

[2] Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG et al. Circulating skeletal stem cells. J. Cell Biol. 2001;153:1133-40.

[3] Bain B. Bone marrow biopsy morbidity: review. J Clin Pathol. 2005;58:406-8.

[4] Zhang M, Huang B. The multi-differentiation potential of peripheral blood mononuclear cells. Stem Cell Res Ther. 2012;3:1-10.

[5] Damon LE, Damon LE. Mobilization of hematopoietic stem cells into the peripheral blood. Expert Rev. Hematol. 2009;2:717-33.

[6] Yoder MC. Human endothelial progenitor cells. Cold Spring Harb Perspect Med. 2012;2:1-14.

[7] Zvaifler NJ, Marinova Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2000;2:477-88.

[8] Costa-Rodrigues J, Teixeira CA, Sampaio P, Fernandes MH. Characterisation of the osteoclastogenic potential of human osteoblastic and fibroblastic conditioned media. J. Cell. Biochem. 2010;109:205-16.

[9] Muto A, Mizoguchi T, Udagawa N, Ito S, Kawahara I, Abiko Y et al. Lineage-committed osteoclast precursors circulate in blood and settle down into bone. J. Bone Miner. Res. 2011;26:2978-90.

[10] Galligan CL, Fish EN. The role of circulating fibrocytes in inflammation and autoimmunity. J Leukoc Biol. 2013;93:45-50.

[11] Bian ZY, Li G, Gan YK, Hao YQ, Xu WT, Tang TT et al. Increased number of mesenchymal stem cell-like cells in peripheral blood of patients with bone sarcomas. Arch Med Res. 2009;40:163-8.

[12] Roufosse CA, Direkze N, Otto W, Wright N. Circulating mesenchymal stem cells. Int J. Biochem. Cell Biol. 2004;36:585-97.

[13] Rochefort GY, Delorme B, Lopez A, Herault O, Bonnet P, Charbord P et al. Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells. 2006;24:2202-8.

[14] Kim S, Honmou O, Kato K, Nonaka T, Houkin K, Hamada H et al. Neural differentiation potential of peripheral blood-and bone-marrow-derived precursor cells. Brain Res. 2006;1123:27-33.

[15] Kassis I, Zangi L, Rivkin R, Levdansky L, Samuel S, Marx G et al. Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. Bone Marrow Transplant. 2006;37:967-76.

[16] Lai Y, Sun Y, Skinner CM, Son EL, Lu Z, Tuan RS et al. Reconstitution of marrow-derived extracellular matrix ex vivo: a robust culture system for expanding large-scale highly functional human mesenchymal stem cells. Stem Cells Dev. 2010;19:1095-107.

[17] Zheng RC, Park YK, Cho JJ, Kim SK, Heo SJ, Koak JY et al. Bone regeneration at dental implant sites with suspended stem cells. J Dent Res. 2014;93:1005-13.

[18] Lee JE, Heo SJ, Koak JY, Kim SK, Han CH. Bone regeneration with rabbit bone marrow-derived mesenchymal stem cells and bone graft materials. Int J oral Maxillofac Implants. 2011;27:1389-99.

[19] Hartman EH, Vehof JW, Spauwen PH, Jansen JA. Ectopic bone formation in rats: the importance of the carrier. Biomaterials. 2005;26:1829-35.

[20] Kim S, Honmou O, Kato K, Nonaka T, Houkin K, Hamada H et al. Neural differentiation potential of peripheral blood- and bone-marrow-derived precursor cells. Brain Res. 2006;1123:27-33.

[21] Chen XD, Dusevich V, Feng JQ, Manolagas SC, Jilka RL. Extracellular Matrix Made by Bone Marrow Cells Facilitates Expansion of Marrow-Derived Mesenchymal Progenitor Cells and Prevents Their Differentiation Into Osteoblasts. J Bone Miner Res. 2007;22:1943-56.

[22] Prewitz MC, Seib FP, von Bonin M, Friedrichs J, Stißel A, Niehage C et al. Tightly anchored tissue-mimetic matrices as instructive stem cell microenvironments. Nat. Methods. 2013;10:788-94.

[23] Chen XD, Akiyama K, You YO, Yamaza T, Chen C, Tang L et al. Characterization of bone marrow derived mesenchymal stem cells in suspension. J Stem Cell Res Ther. 2012;3:40-53.

[24] Dominici M, Le Blanc K, Mueller I, Slaper Cortenbach I, Marini F, Krause D et al. Minimal criteria for defining multipotent mesenchymal

stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315-7.

[25] Cho YJ, Heo SJ, Koak JY, Kim SK, Lee SJ, Lee JH et al. Promotion of osseointegration of anodized titanium implants with a 1α, 25-dihydroxyvitamin D3 submicron particle coating. Int J oral Maxillofac Implants. 2010;26:1225-32.

[26] Lu J, Gallur A, Flautre B, Anselme K, Descamps M, Thierry B et al. Comparative study of tissue reactions to calcium phosphate ceramics among cancellous, cortical, and medullar bone sites in rabbits. J Biomed Mater Res. 1998;42:357-67.

[27] Hardwick R, Dahlin C. Healing pattern of bone regeneration in membrane-protected defects: a histologic study in the canine mandible. Int J oral Maxillofac Implants. 1994;9:13-29.

[28] Terheyden H, Lang NP, Bierbaum S, Stadlinger B. Osseointegration-communication of cells. Clin Oral Implants Res. 2012;23:1127-35.

[29] Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996;17:137-46.

-국문초록-

토끼 혈액 유래 중간엽줄기세포 적용 double-canaled 임플란트 골 재생 연구

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목적: 혈액 채득은 골수를 채득 하는 것 보다 쉽고 안전하며 혈액에는 중간엽줄기세포를 포함 하고 있다. 혈액에서 중간엽줄기세포를 채득하는 법은 여러 가지 있지만 보다 쉽게 채득하는 방법을 개발할 필요성이 있다. 본 연구의 목적은 가토 혈액에서 중간엽줄기세포를 획득하여, *in vitro* 와 *in vivo* 골 형성 능력 특성 분석하고 혈액 중간엽줄기세포를 double-canaled 임플란트에 적용하여 골수 중간엽줄기세포와 골 재생 능력을 비교 평가한다.

재료 및 방법: *혈액 중간엽줄기세포의 획득.* New Zealand white rabbit 을 이용하여 장골과 marginal ear vein 에서 골수 mononuclear cells 와 혈액 mononuclear cells 을 각각 분리 하였다. 골수 mononuclear cells 과 혈액 mononuclear cells 을 Ammonium-Chloride-Potassium lysing buffer 을 이용하여 purified 하였다. Purified 한 골수 mononuclear cell 은 plastic culture plate 에 배양하고 14 일 후 계 대 배양 하였다. 계 대 배양 한 3 passage 인 골수 중간엽줄기세포를 Triton X-100 을 이용하여 extracellular matrix (ECM)-coated culture plate 를 만들었다. Purified 한 혈액

mononuclear cells 은 ECM-coated culture plate 에 배양하고 14 일 후 plastic culture plate 에 계 대 배양 하였다.

In vitro 와 in vivo 에서 혈액 중간엽줄기세포의 특성 분석. Plastic culture plate 에 계 대 배양 후 세포의 morphology, proliferation, 유 세포 분석 및 분화 능력실험을 *in vitro* 에서 진행하여 골수 중간엽줄기세포와 비교 분석하였고 *in vivo* 에서 혈액 중간엽줄기세포의 osteogenic potential 과 immunological reaction 을 immunocompromised mice (BALB/c) 실험을 통해 관찰하였다.

Double-canaled 임플란트에서의 혈액 중간엽줄기세포의 골 재생 능력. 80 개의 double-canaled 임플란트와 20 마리 가토를 준비하였다. 가토에서의 골 재생 능력을 평가하기 위해 defect only 군, HA/TCP 군, 골수 중간엽줄기세포와 혈액 중간엽줄기세포를 전달 체 (HA/TCP)에 부착시킨 군으로 나누었다. 임플란트 식립 시 상부 canal 은 가토 tibia 의 cortical 부분에 위치시키고 하부 canal 은 marrow 부분에 각각 위치 시켰다. 3 주 및 6 주 후 조직학적 및 조직형태학적 검사를 통한 골 재생을 관찰 및 측정하였다.

결과: 혈액 mononuclear cell 을 ECM-coated culture plate 에 배양하는 방법을 통해 혈액 중간엽줄기세포를 성공적으로 획득할 수 있었다. 혈액 줄기세포는 전형적인 fibroblast-like morphology 를 가지고 골수 중간엽줄기세포와 같은 증식 능력을 나타내었다. 혈액 중간엽줄기세포는 중간엽줄기세포 특성인 osteogenic, adipogenic, chondrogenic 분화 능력을 지니고 있었다. 혈액 중간엽줄기세포는 중간엽줄기세포 surface character 인 CD90 에 양성 반응을 보였고 hematopoietic stem cells (HSCs) 특성이 CD14 에서는 음성 반응을 보였다. *In vitro* 와 *in vivo* 실험을 통해서 ECM-coated culture plate 를 통해 획득한 혈액 중간엽줄기세포는 기존의 골수 중간엽줄기세포와 morphology, proliferation 과 differentiation

potential 에서 비슷한 성질을 나타냈다. 조직학적 분석을 통해 혈액 중간엽줄기세포를 double-canaled 임플란트에 적용하여 골 형성을 관찰할 때 BMMSCs 군과 PBMSCs 군에서 osteogenic surface positive cells 를 포함한 mature bone 을 볼 수 있었고 HA/TCP 군에서는 fibrovascular tissue 를 관찰할 수 있었다. Defect only 군에서는 canal 의 lateral side 에서만 골 형성을 관찰할 수 있었다. 6 주에서 3 주보다 더 많은 양의 mature bone 을 볼 수 있었다. 혈액 중간엽줄기세포를 double-canaled 임플란트에 적용한 가토 실험에서의 골 재생 결과를 볼 때 3 주 및 6 주 후 upper canal 부분에서 의 골 재생은 세포를 부착한 군에서 defect only 군 과 HA/TCP 군에 비해 유의하게 높았다 ($p < 0.05$). 3 주 후 lower canal 에서는 네 그룹간의 차이를 보이지 않았으나 ($p > 0.05$) 6 주 후 lower canal 에서는 세포를 부착한 군에서 defect only 군과 HA/TCP 군에 비해 유의하게 높은 골 재생 결과를 보였다 ($p < 0.05$). 하지만 BMMSCs 군과 PBMSCs 군 사이에는 upper canal 과 lower canal 에서 3 주 및 6 주 후 유의한 차이를 보이지 않았다 ($p > 0.05$).

결론: 본 연구에서 중간엽줄기세포를 혈액에서 성공적으로 획득할 수 있었다. 혈액에서 유래된 중간엽줄기세포는 *in vitro* 와 *in vivo* 에서 기존의 골수 중간엽줄기세포와 비슷한 성질을 가지고 있었다. PBMSCs 군과 BMMSCs 군은 비슷한 골 재생 능력을 보여주었다. 이러한 혈액 중간엽줄기세포는 치과임상에서 골 재생을 위한 유망한 소스로 쓰일 수 있음을 이번 실험에서는 보여주었다.

주 요 어: 골수 중간엽줄기세포, 말초혈액, 중간엽줄기세포이식, 치과이식, 기질, 세포 외

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