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혈소판 풍부 피브린이 골재생에 미치는 효과

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The effects of Platelet-Rich Fibrin on the bone regeneration *in vivo and in vitro* study

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ABSTRACT

The effects of Platelet-Rich Fibrin on the bone regeneration *in vivo* and *in vitro* study

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임플란트 식립을 위해서는 우선 골질과 골양이 풍부해야 하는데, 최근 많은 양의 골과 양질의 골을 얻기위해 다양한 연구들이 이루어지고 있다. 골재생시 많은 양의 골과 양질의 골을 얻기 위해서 최근 골 형성을 촉진시키는 물질을 적용하는 시도가 이루어지고 있다. 골 형성을 촉진시키는 물질로는 골유도단백질(bone morphogenic protein, BMP), 혈소판 유래 성장인자(platelet derived growth factor, PDGF), 형질 전환 성장인자(transforming growth factor, TGF)등이 있으며, 이러한 성장인자는 혈소판이 분해되면서 다량 분비하게 된다.

이에 착안하여 본 연구에서는 PRF를 이용하여 PRF내의 성장인자가 조골 세포의 증식과 분화에 어떤 영향을 미치는지 알아보고, 성견의 골 결손부위에 골이식술을 시행했을 때 PRF의 효과를 알아보고자 하였다.

그 결과 PRF는 MG-63세포의 ALP 활동과 석회화 및 광화를 촉진시키며, 골형성과 관계된 생체지표 유전자 역시 증폭되는 것을 확인하였다. 성견에서도 방사선학적, 조직학적 분석을 한 결과 2주군에서 PRF를 섞은 bio-oss를 이식한 실험군 II에서 bio-oss만을 이식한 실험군 I과 대조군에 비해 신생골

형성이 증가되는 것을 관찰하였다.

이에 PRF는 골 재생에 매우 큰 효과가 있다는 결론을 내렸으며, 간단한 방법으로 얻을수 있는 훌륭한 골이식 첨가제라는 것을 보여주었다.

I. Introduction

Dental implant surgery has been commonly practiced on edentulous patients in recent years. Sufficient bone quality and quantity are needed before performing implant placement. Recently, various studies have been conducted to acquire sufficient bone quality and quantity. Autogenous bone grafts, xenografts, allografts, and alloplastic bone grafts have been developed to restore bone defects. Various substances that promote bone formation have also been researched.[1] These substances include bone morphogenic protein (BMP), platelet derived growth factor (PDGF), and transforming growth factor (TGF), which are known to be abundant within platelets.[2] These growth factors promote cell differentiation during the healing process, induce matrix reformation, and promote cell recovery.[3] With the aid of tissue engineering advancements, numerous studies have investigated growth factors, in particular platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). Marx et al.[4] first introduced bone grafting using PRP, and reported that bone maturity was 1.61 - 2.16 times more effective with 15 - 30% bone density improvements. Other studies have also reported the effectiveness of bone grafting using PRP for bone formation.[5-7] However, in contrast, other studies have shown that the effects of PRP on bone regeneration and formation do not considerably differ from those of the control, and that PRP does not improve bone regeneration.[3,8,9] Furthermore, the use of thrombin as a trigger of the coagulation cascade during the production of PRP has been highlighted as an issue. While thrombin is a platelet activating factor that induces the release of growth factors,[10] the use of bovine thrombin has been reported to increase the risk of developing Crutzfeld-Jacob disease and immune reactions, in which

thrombin antibodies are produced.[11,12]

PRF is a modified form of PRP that can be obtained after one cycle of centrifugation without the addition of anticoagulants or heterogeneous thrombin.[13] It is physiological and releases growth factors that are found within platelets, including PDGF, TGF, insulin-like growth factor (IGF), fibroblast growth factor (FGF), and epithelial growth factor (EGF).[14,15] The clinical use of PRF for bone grafting is currently widespread in the field of dentistry. However, research into the effects of PRF growth factors on bone regeneration is insufficient, and, to the best of our knowledge, there has been no examination of a combined *in vitro* and *in vivo* study. Therefore, this study aimed to investigate the effects of PRF growth factors on the proliferation and differentiation on MG-63 osteoblastic cells, and the effectiveness of PRF in the bone grafting of defects in an adult dog, both *in vivo* and *in vitro*.

II. Materials and Methods

II-1. *In vitro* study

II-1-1. Cell culture

Human osteosarcoma cell line MG-63 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured by following the instruction provide from ATCC. Briefly, MG-63 cells were maintained in Eagle's minimum essential medium (EMEM) (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100 μg/ml Streptomycin

and 100 Unit/ml penicillin (Gibco, Grand Island, NY, USA) in a humidified environment at 5% CO_2 and 37°C.

II-1-2. Preparation of Platelet rich fibrin

PRF was processed according to the protocol of Choukroun's PRF.[13] Briefly, blood from 1 non-smoking healthy volunteer without any medical problem was prepared according to the PRF protocol. Briefly, blood samples were taken without an anticoagulant in 10 ml glass-coated plastic tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ, USA) and immediately centrifuged at 2000 rpm for 10 minutes. A fibrin clot formed in the middle part of the tube, while the upper part contained acellular plasma, and the bottom part contained red corpuscles. The fibrin clot was easily separated from the lower part of the centrifuged blood. The PRF clot was gently pressed into a membrane with sterile dry gauze.

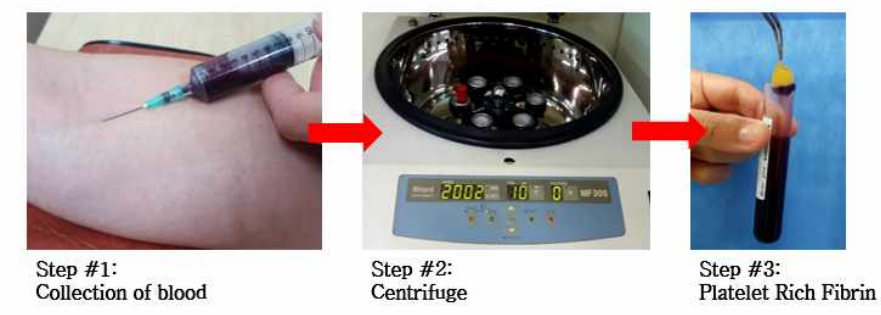

Figure 1. The preparation of platelet rich fibrin from blood.

II-1-3. Cell cytotoxicity assay

To determine the cytotoxicity of PRF in MG-63 cells, the colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed. Briefly, MG-63 cells were cultured at a density of 1 x 10^5 cells/ml in 12-well plates and allowed to attach the well for overnight. After incubation, cultured MG-63 cells were treated with 5, 10, and 20% (w/v) PRF for 24 h. Thereafter, 200 μl of 5 mg/ml MTT (Sigma-Aldrich, St.Louis, MO, USA) was added into cultured FaDu cells and incubated another 4 h. Sequentially, supernatant was removed, and MTT crystal were dissolved in 200 μl/well dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). Finally, optical density was measured at 570 nm by a spectrophotometer (Epoch, Biotek, Winooski, VT, USA).

II-1-4. Cell live & dead assay

To visualize the live and dead MG-63 cell by the treatment of PRF, the cell live and dead assay was performed by green calcein AM and ethidium homodimer-1. Briefly, MG-63 cells were cultured at a density of 1 x 10^5 cells/ml in 8 well chamber slide (Electron Microscopy Sciences, Hatfield, PA, USA) and allowed to attach the well for overnight. After incubation, cultured MG-63 cells were treated with 5, 10, and 20% (w/v) PRF for 24 h. Thereafter, to visualize either live or dead FaDu cells, cell live and dead assay was performed by cell live & dead assay kit (ThermoreFisher Scientific, Rockford, IL, USA), which is composed of green calcein AM to stain the live cells as a green fluorescence and ethidium homodimer-1 to stain the dead cells as a red fluorescence, following the instructions provided by the manufacturer. Cells were

imaged using a fluorescence microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA).

II-1-5. Alkaline phosphatase activity assay

To determine the expression and activity of alkaline phosphatase (ALP) in the MG-63 cells treated with either PRF or bone grafting materials, cells were cultured at a density of 4 X 10^2 cells/ml in 6-well culture plates were maintained with 20% PRF in the serum-free media for 3, 7 and 14 days. Thereafter, alkaline phosphatase was determined by LabAssay™ ALP (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instruction associated with alkaline phosphatase staining. Finally, stained cells were imaged using a microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA). In addition, to measure the activity of alkaline phosphatase in the MG-63 cells treated with either PRF or bone grafting materials, cells were cultured at cultured at a density of 4 X 10^2 cells/ml in 6-well culture plates were maintained with 20% PRF in the serum-free media for 3, 7, and 14 days. Thereafter, the activity of alkaline phosphatase was determined by LabAssay™ ALP (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instruction associated with ELIS Aassay. Finally, the activity of alkaline phosphatase was normalized to total protein concentration and measured at 280 nm wavelength with a NaoDrop ND-1000 spectrometer (NanoDrop Technologies Inc., DE, USA).

II-1-6. Total protein extraction

To normalize the activity of alkaline phosphatase, total proteins were extracted from MG-63 cells. Briefly, culture MG-63 cells were harvested,

lysed using cell lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitor cocktails, and incubated for 1 h at 4° C. Lysates were centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was used as the cytosolic fraction. Total protein concentrations of the cell lysates were determined by bicinchoninic acid protein assays (Thermo Scientific, Rockford, IL, USA).

II-1-7. Alizarin Red S staining

To assess the calcification of MG-63 cells treated with PRF, cells were cultured at a density of 4 X 10^2 cells/ml in 6-well culture plates were maintained with 20% PRF in the serum-free media for 3, 7, and 14 days. Briefly, the culture medium was removed at the end of defined culture periods. Cells were washed with phosphate buffered saline, fixed with ice-cold 70% ethanol for 1 h. and were rinsed with sterile and deionized water. Thereafter, cells were stained with 2% Alizarin Red S solution (Sigma Aldrich, MO, USA) for 30 min at room temperature. The coloring solution was removed and cells were rinsed with deionized water for several times. Finally, stained cells were evaluated by an inverted light microscope for detection of orange-red calcified nodules.

II-1-8. Von Kossa staining

To verify the osteogenic differentiation, Von Kossa staining was performed. Briefly, MG-63 cells were cultured at cultured at a density of 4 X 10^2 cells/ml in 6-well culture plates were maintained with 20% PRF in the serum-free media for 3, 7, and 14 days. Thereafter, cells were rinsed with PBS (Sigma Aldrich, MO, USA) and were fixed with ice-cold 4% paraformaldehyde (Sigma Aldrich, MO, USA) for 20 min. After washing with deionized water, 1% $AgNO_3$ (Gibco, MA, USA) was added into the cells and were exposed to UV radiation for 20 min. After washing the cells with deionized water for several times, cells were treated with thiosulfate for 5 min in order to remove detached Ag^+ ions. Repeating the washing step using deionized water, the area containing phosphate salts were detected by the appearance of black nodules. Finally, stained cells were imaged using a fluorescence microscopy (EclipseTE2000, Nikon Instruments, Melville, NY, USA).

II-1-9. Quantitative polymerase chain reaction (qPCR)

To verify the alteration of biomarkers genes associated with bone formation at the mRNA level, MG-63 cells were cultured with 20% PRF (w/v) in the serum-free media for 3, 7, and 14 days. MG-63 cells were harvested at the end of defined culture periods and were washed with diethyl pyrocarbonate (DEPC) water. Thereafter, total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into first-strand cDNA using the ThermoScript RT-PCR system (Life Technologies). For qPCR, cDNA was amplified using a SureCycler 8800 (Agilent Technologies, Santa Clara, CA, USA) and 2X TOPsimple DyeMIX-nTaq

(Enzynomics, Seoul, Korea), according to the instructions of the manufacturers. Gene expression was determined by agarose gel electrophoresis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization. After PCR, amplified PCR products were visualized by DNA agarose gel electrophoresis. The differences in expression levels are presented as histograms after densitometry analysis using a VersaDoc imaging system (Bio-Rad, Hercules, CA, USA).

Table 1. Primer sequence for qPCR to verify the biomarker genes associated with bone formation

Gene	Primer Sequences	PCR product size (bp)	NCBI accession #
Human Collagen Type I	Forward : 5'-ACGAAGACATCCCACCAATC-3' Reverse: 5'-AACCTCTGTGTCCCTTCATTC-3'	714	NM_000088.3
Human bone morphogenetic protein-2	Forward : 5'-CTACCAGAAACGAGTGGGAAA-3' Reverse : 5'-CACGTACAAAGGGTGTCTCTTA-3'	549	NM_001200.3
Human osteocalcin	Forward : 5'-TCACACTCCTCGCCCTATT-3' Reverse : 5'-TGAAAGCCGATGTGGTCAG-3'	259	NM_199173.5
Human GAPDH	Forward : 5'-GATTCCACCCATGGCAAATTC-3' Reverse : 5'-GTCATGAGTCCTTCCACGATAC-3'	380	NM_001289746.1

II-2. *In vivo* study

II-2-1. Generation of animal models

The procedures used in present study were in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC2015-A0041) at Chosun University. Six adult mongrel dogs, weighing approximately 15 kg each, were used. General anesthesia of the adult dogs was performed by injecting a mixture of 1.5 ml 2% zylazine hydrochloride (3 mg/kg, Rumpun, Bayer Korea Ltd., Korea) and zolazepam(10 mg/kg, Zoletil 50, Virbac Lab., France). First, 10 ml blood from cephalic vein were prepared according to the PRF protocol. PRF was processed according to the protocol of Choukroun's PRF.[13] Sequentially, the femoral bone was exposed and the intentional defects were made. The defects was performed three 8-mm diameter in both femoral bone by trephine drill. The defect divided in three group : (1) defect left unfilled (control group), (2) defect filled with only 0.25 g Bio-oss (Geistlich, Wolhusen, Switzerland) (experimental group I) and (3) defect filled with 0.25 g Bio-oss (Geistlich, Wolhusen, Switzerland) mixed PRF (experimental group II). After careful filling of the defects, Layer-to-layer suture was performed completely. After healing periods of 2 and 4 weeks, experimental animals were sacrificed. All biopsies were immersed in 10% neutral-buffered formaldehyde.

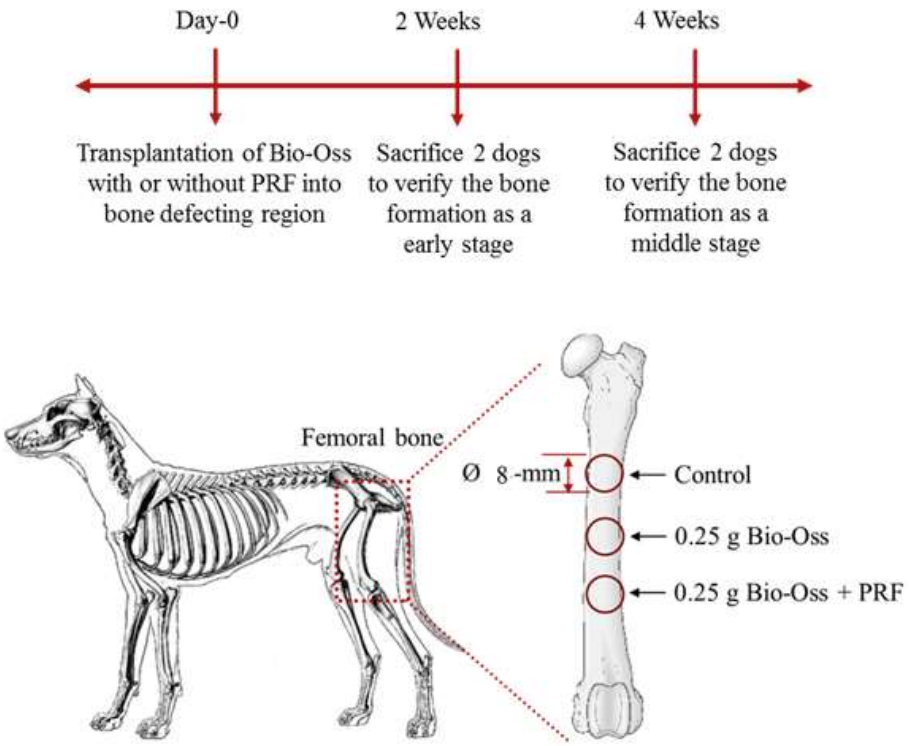

Figure 2. The schematic diagram of *in vivo* study using animal models generated by femoral bone defects

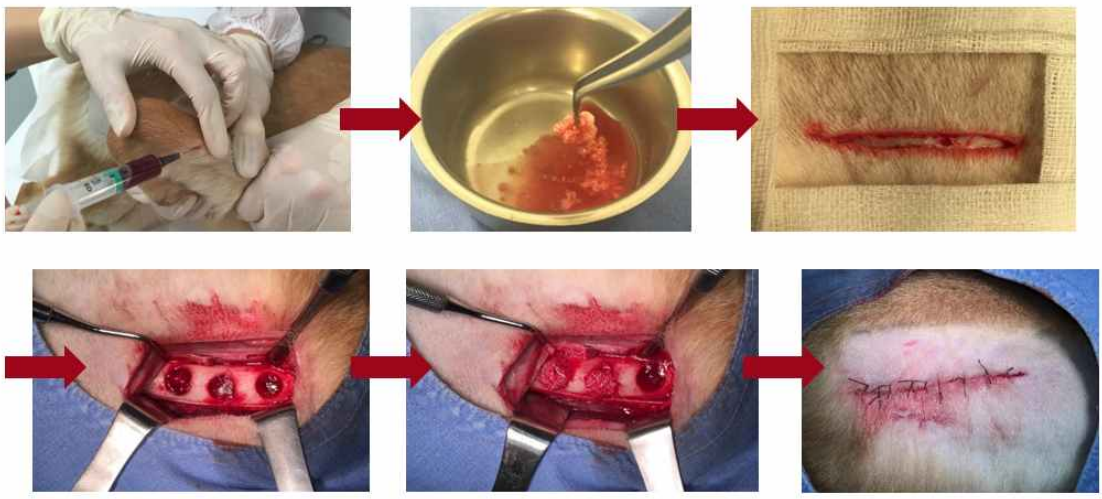

Figure 3. Defect filling procedure *in vivo* study

II-2-2. Histological assessment

At the end of defined culture periods, animal models were sacrificed according to the permissioned protocol by IACUC at Chosun University. Thereafter, femoral bones were dissected, washed using PBS, and were fixed with 4% paraformaldehyde for 72 h at 4°C. Sequentially, dissected femoral bone were performed the post-fixation using 4% paraformaldehyde for 48 h at 4°C. Fixed femoral bones were decalcified by 1 mM ethylenediamine-tetraacetic acid (EDTA, pH 7.4) solution, which was changed every 5 days. At the end of decalcification process, femoral bones were performed paraffin embedding. Serial femoral bone sections of exact 5 μm thickness were obtained to prepare slides. Hematoxylin & eosin staining was performed to verify the morphological alteration of bone defecting region.

II-2-3. Micro-CT assessment

Structural alterations of femoral bone architecture were evaluated by micro-CT scanning. Freshly dissected tibias and femurs were immediately fixed in 4% paraformaldehyde, followed by micro-CT imaging analyses performed at the Korea Basic Science Institute at Gwangju, Republic of Korea, using Quantum GC microCT Imaging System (Perkin Elmer, Shelton, CT, USA).

III. Results

III-1. *In vitro* study

III-1-1. The viability of MG-63 cells treated with PRF

To verify the biological safety of PRF, MG-63 cells were cultured with 5, 10, and 20% (w/v) PRF in the serum-free media for 24 h. Thereafter, MTT assay was performed to measure the cytotoxicity as shown in Fig. 4. The cell viabilities of MG-63 cells were verified as 83 ± 6.1%, 95 ± 6.8% and 98 ± 5.9%, at 5, 10, and 20% (w/v) PRF compared with the control, which was cultured in the culture media containing 10% FBS. Therefore, the viabilities of MG-63 cells treated with 10 and 20 % (w/v) PRF in the serum-free media did not have a statistical significance with that of control.

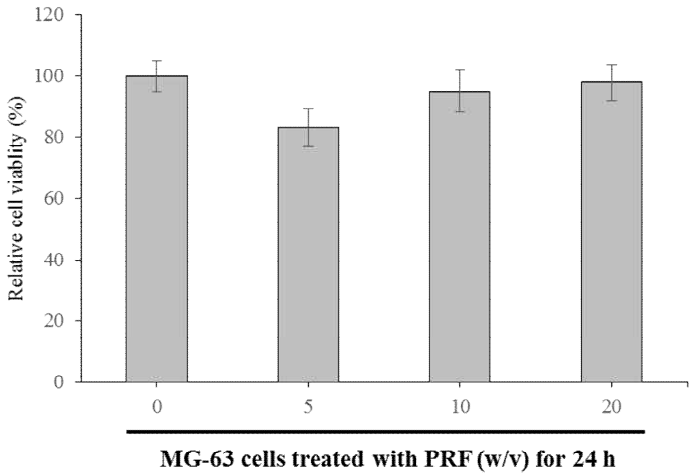

Figure 4. The cytotoxicity of PRF in MG-63 cells. MG-63 cells were treated with 5, 10, and 20% PRF in the serum-free media for 24 h. Thereafter, MTT assay was performed to verify the cytotoxicity of PRF.

III-1-2. The survival rate of MG-63 cells treated with PRF

To confirm the biological safety of PRF, cell live and dead assay (ThermoreFisher Scientific, Rockford, IL, USA) was performed according to the instruction supplied from manufacture. As shown in Fig. 5, almost MG-63 cells were stained as a green fluorescence through the cleavage of green calcein AM by esterase in the live cells. Although the population stained as a red fluorescence by ethidium homodimer-1 in dead cells were slightly increased in the MG-63 cells treated with 5% PRF for 24 h, almost MG-63 cells were stained as a green fluorescence as well as control, which was cultured in the culture media containing 10% FBS. Therefore, these data demonstrate that PRF has a biological safety in the MG-63 cells. Furthermore, the optimal PRF condition was estimated by 10% (w/v) in the serum free media.

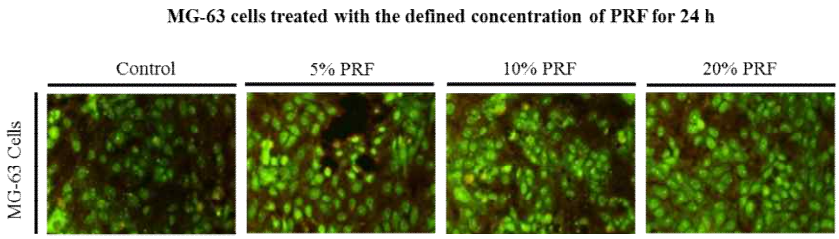

Figure 5. The MG-63 cell survival in PRF. MG-63 cells were treated with 5, 10, and 20% PRF in the serum-free media for 24 h. Thereafter, cell live and dead assay was performed by cell live and dead assay kit composed of green calcein AM to stain the live cell as a green fluorescence and ethidium homodier-1 to stain the dead cell as a red fluorescence. Cells were imaged using a fluorescence microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA).

III-1-3. The assessment of alkaline phosphatase activities in the MG-63 cells cultured with 10% (w/v) PRF

Alkaline phosphatase is a well-known biomarker associated with bone formation. Therefore, to verify the new bone formation in the MG-63 cells cultured with 10% (w/v) PRF, alkaline phosphatase staining assay was performed. As shown in Fig. 6A, the stained signals of alkaline phosphatase were gradually increased in the MG-63 cells treated with PRF as a time-dependent manner compared with control. Furthermore, to measure the alkaline phosphatase activity, alkaline phosphatase activity assay was performed as shown in Fig. 6B. At the 3 day of culture period, relative alkaline phosphatase activity was verified by approximately 157.5 ± 28.35 in the MG-63 cells cultured with 10% PRF compared with control (100 ± 8). Therefore, the alkaline phosphatase activity of MG-63 cells treated with 10% PRF was approximately 0.5 folds higher than control.

Moreover, relative alkaline phosphatase activities were verified by approximately 170 ± 20.4 and 353.3 ± 88.3 in the control and MG-63 cells treated with 10% PRF at 7 day of culture period, respectively, compared with control at 3 day of culture period. Therefore, the alkaline phosphatase activity of MG-63 cells treated with 10% PRF was approximately over 2 folds higher than control at 7 day of culture period. However, relative alkaline phosphatase activities were verified by approximately 260 ± 31.2 and 494.3 ± 31.2 in the control and MG-63 cells treated with 10% PRF at 14 day of culture period, respectively, compared with control at 3 day of culture period. Therefore, the alkaline phosphatase activity of MG-63 cells treated with 10% PRF was approximately 2 folds higher than control at 14 day of culture period. These data consistently indicate that PRF accelerate the new bone formation in the MG-63 cells.

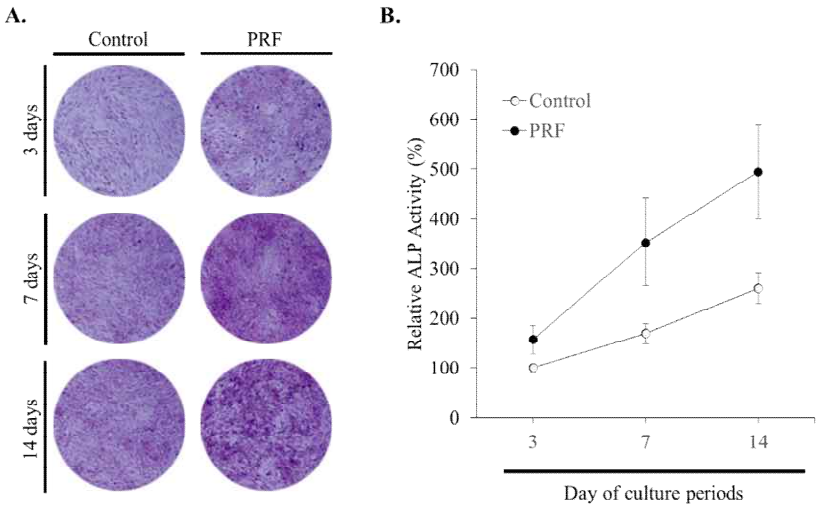

Figure 6. The alkaline phosphatase activity was increased in MG-63 cell treated with 10% (w/v) PRF. MG-63 cells were treated with 10% PRF in the serum-free media for 3, 7 and 14 days. At the end of culture periods, alkaline phosphatase staining and activity assay were performed according to the manufacturer's protocols. Cells were imaged using a fluorescence microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA). The activities of alkaline phosphatase were measured by a spectrophotometer (Epoch, Biotek, Winooski, VT, USA).

III-1-4. The assessment of calcium deposition in the MG-63 cells cultured with 10% (w/v) PRF

New bone formation is closely associated with the calcification on the osteoblast cells. Therefore, to verify the calcification in the MG-63 cells treated with 10% (w/v) PRF, alizarin Red staining was performed. As shown in Fig. 7A, MG-63 cells treated with 10% PRF were significantly stained as a red color by alizarin Red more than control. Furthermore, the population stained as a red color were gradually increased in the MG-63 cells treated with 10% PRF, as a time dependent manner. Furthermore, the staining thresholds of alizarin red were measured shown in Fig. 7B. At the 3 day of culture period, relative alkaline phosphatase activity was verified by approximately 173.5 ± 12.1 in the MG-63 cells cultured with 10% PRF compared with control (100 ± 5.3). Therefore, the mineralization of MG-63 cells treated with 10% PRF was approximately 0.7 folds higher than control.

Moreover, relative staining thresholds of alizarin red were verified by approximately 120 ± 30 and 248 ± 19.8 in the control and MG-63 cells treated with 10% PRF at 7 day of culture period, respectively, compared with control at 3 day of culture period. Therefore, the alkaline phosphatase activity of MG-63 cells treated with 10% PRF was approximately 2 folds higher than control at 7 day of culture period. However, relative staining thresholds of alizarin red were verified by approximately 145 ± 13 and 295 ± 11.8 in the control and MG-63 cells treated with 10% PRF at 14 day of culture period, respectively, compared with control at 3 day of culture period. Therefore, the calcification of MG-63 cells treated with 10% PRF was approximately 2 folds higher than control at 14 day of culture period. These data consistently indicate that PRF accelerates the calcification associated with new bone formation in the MG-63 cells.

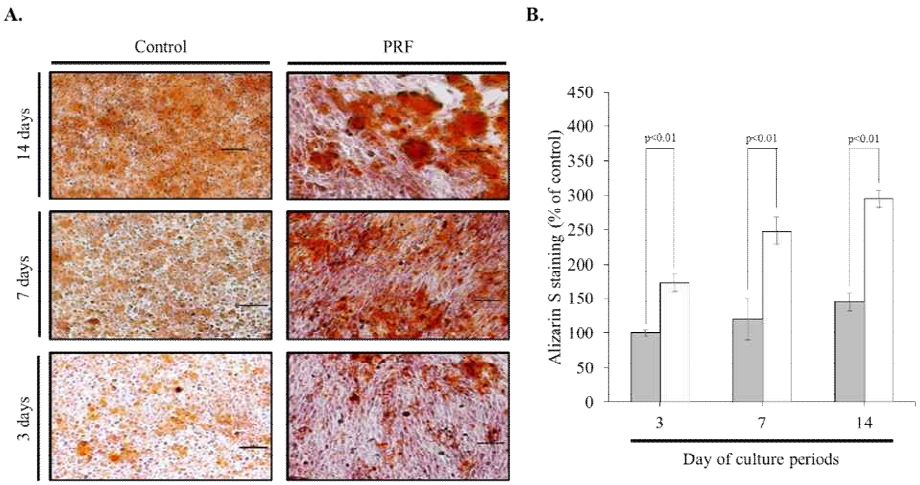

Figure 7. PRF accelerates the calcification on the MG-63 cells. MG-63 cells were treated with 10% PRF in the serum-free media for 3, 7 and 14 days. At the end of culture periods, alizarin red staining and activity assay were performed according to the manufacturer's protocols. Cells were imaged using a fluorescence microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA). The activities of alizarin red staining were measured by a spectrophotometer (Epoch, Biotek, Winooski, VT, USA).

III-1-5. The assessment of mineralization in the MG-63 cells cultured with 10% (w/v) PRF

New bone formation need to the mineralization in the osteoblasts. Therefore, to verify the mineralization in the MG-63 cells treated with 10% PRF, von Kossa staining was performed. As shown in Fig. 8, the staining activity associated with mineralization was significantly higher in the MG-63 cells treated with 10% PRF more than control. Furthermore, the number of formed nodules were verified as approximately 168 ± 38 and 472 ± 56 per culture plates (6 well) in the MG-63 cells without or with 10% PRF, respectively, at the 14 days of culture periods. There data consistently indicate that the PRF accelerate the new bone formation through the increase of mineralization in the MG-63 cells.

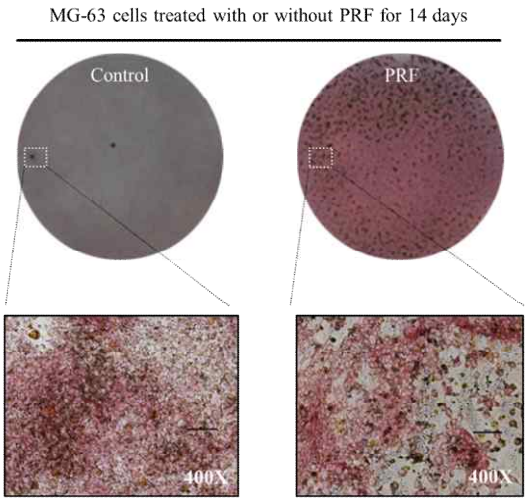

Figure 8. PRF accelerates the mineralization on the MG-63 cells. MG-63 cells were treated with 10% PRF in the serum-free media for 14 days. At the end of culture periods, von Kossa staining were performed according to the manufacturer's protocols. Cells were imaged using a fluorescence microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA).

III-1-6. PRF upregulates the biomarker genes associated with bone formation in the MG-63 cells.

Next, to confirm the acceleration of new bone formation in the MG-63 cells treated with 10% (w/v) PRF, the alteration of biomarker genes associated with new bone formation such as collagen type I, bone morphogenetic protein 2, and osteocalcin at the mRNA level was performed by qPCR. MG-63 cells were treated with 10% (w/v) PRF for 3, 7 and 14 days. Thereafter, at the end of defined culture periods, cells were harvested and total RNA was extracted by TriZol reagent. Sequentially, cDNA was synthesized from 1 μg total RNA after the measurement of concentration of total RNA.

However, collagen type I is a well-known bone matrix component. Therefore, the alteration of collagen type I mRNA was observed as shown in the Fig. 9. The mRNA level of collagen type I were consistently higher in the MG-63 cells treated with 10% (w/v) PRF than control at the all of culture periods. Furthermore, the induction levels of collagen type I mRNA were verified approximately 40.7 ± 7%, 62 ± 8.1%, 91 ± 8.8% higher than control at the 3, 7, and 14 days of culture periods, respectively.

Furthermore, bone morphogenetic protein-2 is a well-known anabolic protein associated with the synthesis of bone matrix. As shown in the Fig. 10, the induction of bone morphogenetic protein-2 mRNA were significantly higher in the MG-63 cells treated with 10% (w/v) PRF than control at the all of culture periods. The induction levels of bone morphogenetic protein-2 mRNA were verified approximately 8.5 ± 0.6, 8.9 ± 1.2, 14.3 ± 2.9 folds higher than control at the 3, 7, and 14 days of culture periods, respectively.

Osteocalcin is a well-known bone matrix component as well as collagen type I. Interestingly, the induction level of osteocalcin mRNA had no significance compared with controls at the 3 and 7 days culture periods. Otherwise, the induction of osteocalcin mRNA were highly increased at the late stage (14 days) of new bone formation in the MG-63 cells treated with 10% (w/v) PRF. However, the induction levels of osteocalcin mRNA were verified approximately 3.3 ± 0.2 folds higher than control at the 14 days of culture periods, respectively.

Taken together, these data consistently indicate that PRF accelerates the new bone formation through the upregulation of biomarker genes associated with bone formation such as collagen type I, BMP-2 and osteocalcin in MG-63 cells.

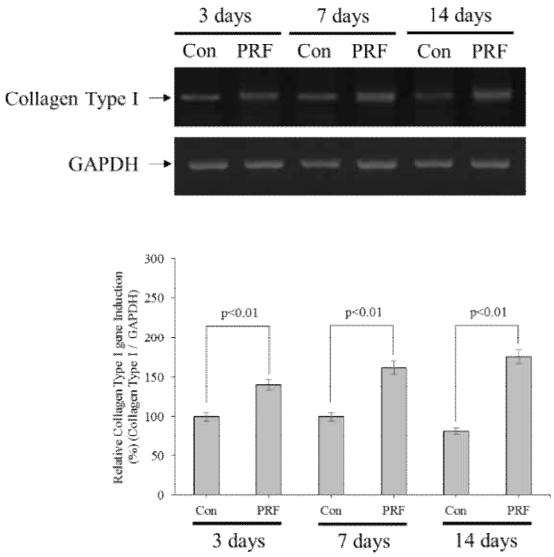

Figure 9. PRF upregulates the collagen type I mRNA in the MG-63 cells. MG-63 cells were treated with 10% PRF in the serum-free media for 3, 7, and 14 days. At the end of culture periods, MG-63 cells were harvested. Sequentially, cDNA was synthesized from total RNA by reverse transcriptase-PCR. Thereafter, quantitative-PCR was performed using collagen type I specific primers.

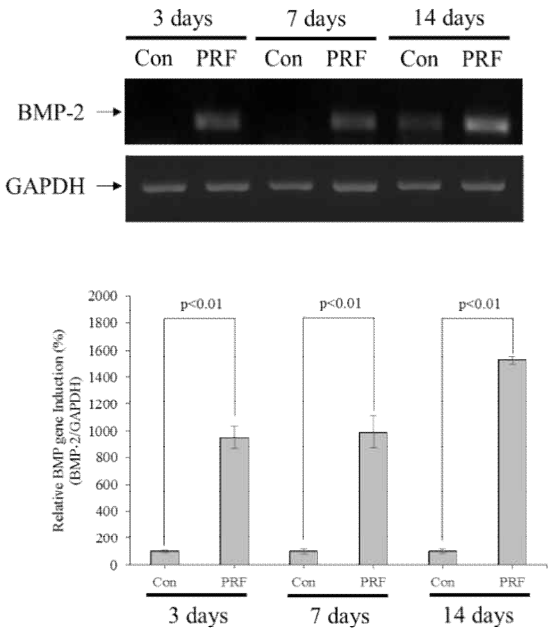

Figure 10. PRF upregulates the bone morphogenetic protein-2 mRNA in the MG-63 cells. MG-63 cells were treated with 10% PRF in the serum-free media for 3, 7, and 14 days. At the end of culture periods, MG-63 cells were harvested. Sequentially, cDNA was synthesized from total RNA by reverse transcriptase-PCR. Thereafter, quantitative-PCR was performed using bone morphogenetic protein-2 specific primers.

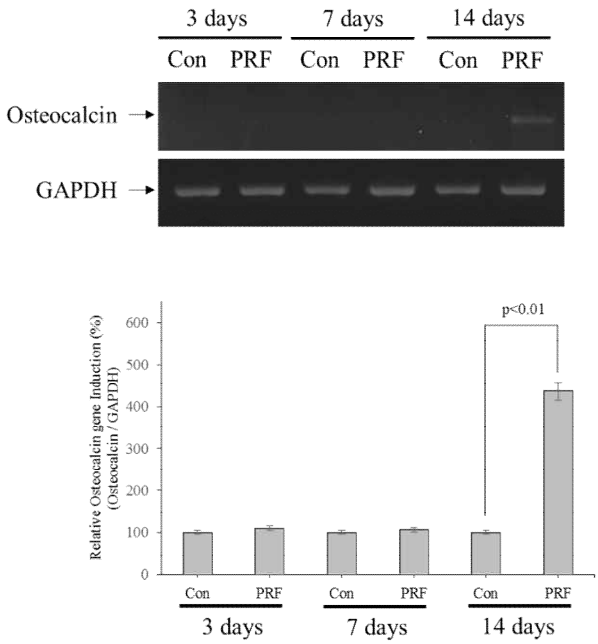

Figure 11. PRF upregulates the osteocalcin mRNA at the late stage of new bone formation in the MG-63 cells. MG-63 cells were treated with 10% PRF in the serum-free media for 3, 7, and 14 days. At the end of culture periods, MG-63 cells were harvested. Sequentially, cDNA was synthesized from total RNA by reverse transcriptase-PCR. Thereafter, quantitative-PCR and quantitative real time-PCR was performed using osteocalcin specific primers.

III-1-7. Bio-Oss used as a bone grafting material does not increase the ALP activities in the MG-63 cells.

To determine whether Bio-Oss used as a bone grafting materials in present study increase the expression of alkaline phosphatase or not, MG-63 cells were cultured with 0.1 g Bio-Oss in the media containing 10% FBS for 3, 7, and 14 days. At the end of culture periods, alkaline phosphatase activity assay was performed according to the manufacturer's protocol. As shown in Fig. 12, relative activities of ALP were measured as 100 ± 16% and 92 ± 12.4% in the MG-63 cells cultured without or with 0.1 g Bio-Oss, respectively, at the 3 days of culture periods. Furthermore, at the 7 days of culture periods, relative activity of ALP was measured as 114 ± 14.7% in the MG-63 cells treated with 0.1 g Bio-Oss compared to control (104 ± 19.2%). However, there was no statistical significance between control and MG-63 cells treated with Bio-Oss at the 7 days of culture periods. Moreover, relative activities of ALP were measured as 102 ± 24% and 108 ± 13.6% in the MG-63 cells cultured without or with 0.1 g Bio-Oss, respectively, at the 14 days of culture periods. There was no statistical significance between control and MG-63 cells treated with Bio-Oss at the 14 days of culture periods. Therefore, taken together, these data demonstrate that the Bio-Oss used as a bone grafting materials may not increase the new bone formation in the MG-63 cells.

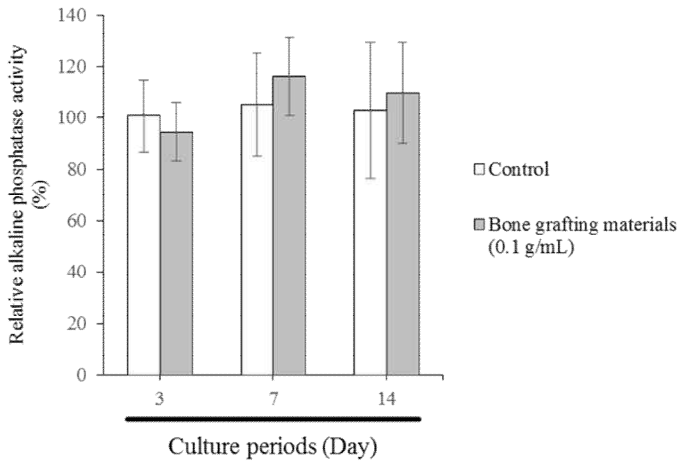

Figure 12. Bio-Oss used as a bone grafting materials dose not increase the alkaline phosphatase activity in the MG-63 cells. MG-63 cells were cultured with 0.1 g Bio-Oss in the media containing 10% FBS for 3, 7, and 14 days. At the end of culture periods, alkaline phosphatase activity assay was performed according to the manufacturer's protocols. The activities of alkaline phosphatase were measured by a spectrophotometer (Epoch, Biotek, Winooski, VT, USA).

III-2. *In vivo* study

III-2-1. Comparative radiographic assessment of new bone formation between PRF accelerates the new bone formation at the defecting bone regions transplanted with bone grafting materials

To verify whether PRF accelerate the new bone formation at the defecting region of bone transplanted with Bio-Oss used as a bone grafting materials, Bio-Oss was transplanted into the defecting region formed on the femoral bone of experimental animals in presence or absence of PRF. Thereafter, experimental animals were dissected at 2 and 4 weeks after transplantation to perform the radiographic analysis associated with bone formation at the defecting region of bone. As shown in Fig. 13 (Left panel), although the defecting region of bone was still observed in the control group, both defecting regions of experimental group-I and -II were stably filled with Bio-Oss used as a bone grafting materials at the 2 weeks after transplantation. Whereas, the defecting region of femoral bone was filled with new bone in the control group at the 4 weeks. However, the amount of Bio-Oss transplanted without PRF into the defecting region of femoral bone reduced in the experimental group-I at the 4 weeks after transplantation. On the other hands, Bio-Oss transplanted with PRF stably remained at the defecting region of femoral bone in the experimental group-II at the 4 weeks. Next, to evaluate the new bone formation at the defecting region of femoral bone transplanted with Bio-Oss in the presence or absence of PRF, bone density analysis was performed as shown in Fig. 13 (Right panel). The bio-Oss transplanted into the defecting region of femoral bone was detected as highlighted blue that indicated the bone with high density. However, natural bones with lighter blue color compared with that of Bio-Oss were significantly higher in the experimental group-II than experimental group-I at both 2 and 4 weeks. Therefore, these data indicate that PRF accelerates the new bone formation in the defecting region of bone transplanted with Bio-Oss.

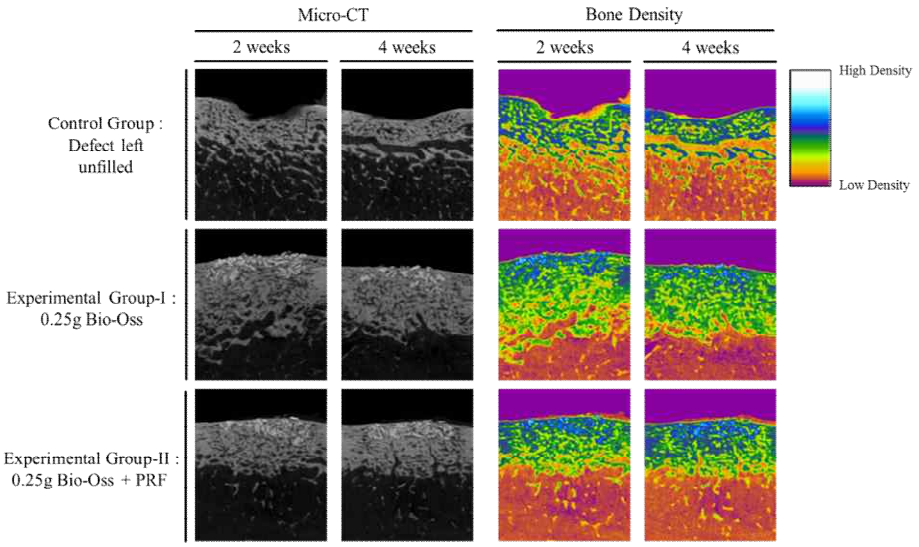

Figure 13. Radiographic analysis show that PRF accelerate the new bone formation at the defecting region of bone transplanted with Bio-Oss. Experimental animals with the Ø8-mm defect on the femoral bone were generated by the protocols permitted from IACUC. Thereafter, 0.25 g Bio-Oss were transplanted into the defecting region of femoral bone in presence or absence of RFP. Femoral bones were dissected from experimental animals at 2 and 4 weeks later to perform the radiographic analysis using micro-CT and bone formation analysis using CTAn image software (Skyscan).

III-2-2. Comparative histological assessment of new bone formation between PRF accelerates the new bone formation at the defecting bone regions transplanted with bone grafting materials

Next, to verify the PRF-induced acceleration of new bone formation at the defecting region of femoral bone transplanted with Bio-Oss in presence of PRF, hematoxylin and eosin staining as a histological assessment was performed as shown in Fig. 14. In the experimental group-I, Bio-Oss transplanted into defecting region of femoral bone acted only as a scaffolds without new bone formation at 2 and 4 weeks after transplantation. Whereas, in the experimental group-II, newly formed bones were increased around the Bio-Oss transplanted into defecting region of femoral bone at 2 weeks after transplantation. However, although newly formed bones were observed around the Bio-Oss transplanted into defecting region of femoral bone at 4 weeks after transplantation, but it was similar with that of 2 weeks. Therefore, these data indicate that PRF accelerates the new bone formation compared with transplantation of Bio-Oss only.

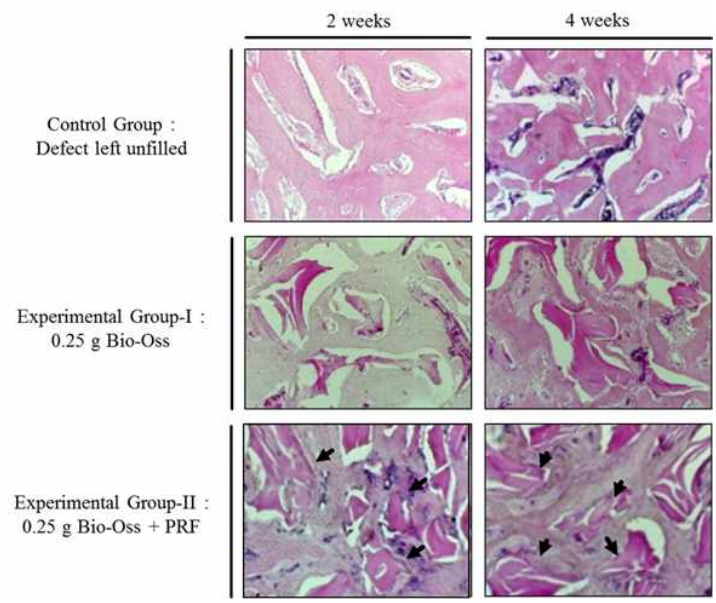

Figure 14. Histologic analysis show that PRF accelerate the new bone formation at the defecting region of bone transplanted with Bio-Oss. Experimental animals with the Ø8-mm defect on the femoral bone were generated by the protocols permitted from IACUC. Thereafter, 0.25 g Bio-Oss were transplanted into the defecting region of femoral bone in presence or absence of PRF. Femoral bones were dissected from experimental animals at 2 and 4 weeks later to perform Hematoxylin & Eosin staining.

IV. Discussion

Bone grafts can promote successful bone regeneration through three main stages: osteogenesis, osteoinduction, and osteoconduction.[16] Currently, the only bone graft material that has osteogenic activities are autogenous bone grafts. Autogenous bone grafts are commonly used and have been considered the gold standard for a long time. However, they are disadvantaged by donor site formation and rapid absorption.[16] Osteoconduction is the physical property of most bone graft materials that serve as a scaffold for new bone growth, such as autogenous bone grafts, allografts, alloplastic bone grafts, and even the xenograft, Bio-oss (Geistlich, Wolhusen, Switzerland) used in this study. Autogenous bone grafts and allografts, which were used to investigate the process by which osteoinduction promotes osteogenesis in the current study, both possess osteoinductive activities.[17] Demineralization of allografts leads to elimination of mineralized bone graft materials. As a result, growth factors that are present within the materials, in particular BMP, are exposed, leading to increased osteoinduction.[18,19]

Growth factors such as BMP are important factors in bone regeneration. Growth factors also include PDGF, TGF, IGF, FGF, and EGF. They are known to promote angiogenesis during the healing process, promote cell differentiation, and contribute to matrix synthesis and formation of new bone by osteoblasts.[2,15,20,21] Howell et al.[22] reported superior bone regeneration in periodontitis patients treated with PDGF and IGF compared with a control group treated with membranes only. Lynch et al.[23] reported significant differences in bone formation around the peri-implant between an experimental group, treated with PDGF and IGF, and a control group during bone grafting for implant placement in adult dogs. In addition, Mohammed et al.[24] reported significant bone regeneration in an experimental group that was treated with TGF-β during *in vivo*-guided tissue regeneration. IGFs are abundant within platelets, and are known to act on osteoblasts in the endosteum to induce their proliferation, form new bone and tissue, and regenerate damaged cells.[25] Vascular endothelial growth factor (VEGF) increases plasma protein penetration in the capillaries, maintains survival of new blood vessels, and induces cell proliferation and differentiation.[26] Due to these advantages, there have been efforts to use

autologous growth factors in bone grafting. Production and clinical use of PRF have also begun. Extensive research is currently being conducted on this topic.[13,27]

PRF is a gel-type fibrin network that contains a high concentration of growth factors; it is obtained after centrifugation of autologous blood.[15] As mentioned in the introduction, the coagulation process is initiated quickly in PRP due to the artificially added thrombin, whereas it occurs gradually and naturally in PRF without additives. Through the gradual coagulation process, PRF obtains a three-dimensional organization that has a higher intensity than a natural fibrin clot.[15] Its organization is in the form of equilateral junctions, and it forms a highly elastic matricial architecture that may be responsible for the longer release time of various growth factors.[15]

Numerous studies have reported the effectiveness of PRF in bone regeneration.[27-29] Choukroun et al.[27] failed to find a quantitative difference between an experimental group, in which allografts containing PRF were used, and a control group during maxillary sinus elevation, but around 4 months of faster bone maturity was recorded in the experimental group by analyzing histological examination results. Moreover, Diss et al.[28] performed transplantation using PRF only during maxillary sinus elevation and reported an increase in the internal height of the maxillary sinus by 3.2 ± 1.5 mm compared with the pre-operation height. He et al.[29] observed proliferation and differentiation of rat osteoblasts to compare between PRF and PRP, and reported superior results with PRF for both ALP expression and induction of mineralization. However, the findings of a considerable number of studies have contradicted these results.[30,31] Gürbüzer et al.[30] investigated osteoblastic activity after applying PRF to the extraction socket, and reported that PRF did not affect bone healing. Zhang et al.[31] also reported a lack of significant difference between a group that was treated with PRF and bovine bone minerals, and a group that was treated with bovine bone minerals only, during maxillary sinus elevation. In this study, *in vitro* and *in vivo* experiments were performed while taking into account the results of these previous studies. In the *in vitro* experiment, ALP activity increased in MG-63 cells treated with PRF 10% (w/v). PRF accelerates the calcification and mineralization of MG-63 cells. It upregulates the biomarker genes

such as collagen type I, bone morphogenetic protein 2, and osteocalcin, which are associated with bone formation in MG-63 cells. The present findings indicate that PRF has a significant effect on bone regeneration. In the *in vivo* experiment, bone formation was greater in Group II than in Group I and the control 2 weeks postoperatively.

In addition to the aforementioned growth factors, PRF also contains the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, as well as IL-4, an anti-inflammatory cytokine.[32] This suggests that PRF may inhibit early stages of inflammation postoperatively and contribute to early stages of bone formation. This was supported by the present *in vivo* study, in which greater bone formation was observed in Group II compared with Group I and the control 2 weeks postoperatively.

These findings demonstrate that PRF has a significant effect on bone regeneration, and it is a safe and effective additive material for bone grafts that is also easy to acquire. However, since differences in the degree of bone formation were observed among different groups during the early stages of bone formation in the in vivo experiment (at 2 weeks), additional studies such as PRF ingredient analyses are required

V. Conclusion

This study investigated the effects of PRF growth factors on the proliferation and differentiation of MG-63 cells *in vitro* study. An *in vivo* experiment was also performed, in which bone graft materials mixed with PRF were implanted onto a femoral bone defect in an adult dog. The following conclusions were drawn:

1) *In vitro* study, The ALP activity was increased in MG-63 cell treated with 10% (w/v) PRF. PRF accelerates the calcification and mineralization on the MG-63 cells. PRF upregulates the biomarker genes such as collagen type I, bone morphogenetic protein 2, and osteocalcin which are associated with bone formation in the MG-63 cells.

2) *In vivo* study, Both radiographical and histological evaluation showed that the new bone formations were significantly increased in the defecting bone region transplanted with Bio-oss and PRF compared with Bio-oss only at 2 weeks after transplantation.

In conclusion, this study demonstrates that PRF has a great effect on bone regeneration and is valuable in clinical use.

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