치의과학과 박사논문

Improvement of the osteogenic potential of BMP-2-/EGCG-coated biphasic calcium phosphate bone substitute

BMP-2-/EGCG로 표면처리된 이상인산칼슘 골이식재의 골형성능 개선 평가

2021년 2월

서울대학교 대학원

치의과학과 치주과학 전공

황 재 호

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이 논문을 치의과학 박사학위논문으로 제출함

2020년 12월

서울대학교 대학원
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Abstract

Improvement of the osteogenic potential of BMP-2-/EGCG-coated biphasic calcium phosphate bone substitute

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Objectives: The aim of this study was to evaluate the enhancement of osteogenic potential of biphasic calcium phosphate (BCP) bone substitute coated with Escherichia coli-derived recombinant human bone morphogenetic protein-2 (ErhBMP-2) and epigallocatechin-3-gallate (EGCG).

Methods: The differentiation and mineralization of osteoblasts were verified with ErhBMP-2-/EGCG solution. After inspecting the coated BCP surface, the cell viability of human mesenchymal stem cells was evaluated in the coated BCP. In the maxillary sinus of 10 male New Zealand white rabbits, a standardized

6-mm diameter defects was bilaterally generated. After removing the bone window and lifting the sinus membranes, BCP coated with ErhBMP-2-/EGCG was applied to one defect of the test group. Uncoated BCP was applied to other defects to form the control group. After 4 or 8 weeks of surgery, animals were sacrificed, and the grafted material and surrounding tissues were subjected to biopsy and histometric analysis.

Results: In the result using osteoblasts, no difference was found between only ErhBMP-2 group and ErhBMP-2-/EGCG group. There was no difference between coated BCP and non-coated BCP with human mesenchymal stem cells. In the *in vivo* study, the 4 and 8-week test group showed more new bones (%) than the control group (P<0.05). The 8-week test group showed more new bones (%) than the 4-week test group (P<0.05).

Conclusion: ErhBMP-2-/EGCG-coated BCP was effective as a bone graft material, showing enhanced osteogenic potential and minimal side effects in a rabbit sinus augmentation model.

Keywords: biphasic calcium phosphate; bone morphogenetic protein 2; bone substitute; epigallocatechin-3-gallate

Student number: 2016-31771

CONTENTS

I. INTRODUCTION

Bone morphogenetic protein (BMP) plays important role in bone remodeling[1]. BMP has an osteoinductive activity and induces differentiate mesenchymal stem cells into osteoblastic cells for bone formation[2]. Previous study showed that skeletal deformation and osteoporosis were related to dysregulation of BMP signaling[3]. Various types of BMP (rhBMP-2, -4, -6, -7 and -9) were widely used in regenerative therapies[4]. However, due to high cost and low production, the use of BMP is limited.

Escherichia coli-derived recombinant human bone morphogenetic protein-2 (ErhBMP-2) has been used as a substitute for rhBMP-2, which is derived from BMP gene-transfected Chinese hamster ovary cells [5-8]. ErhBMP-2 could overcome the limitations (high cost and low yield) of rhBMP-2, which is produced in mammalian cells. ErhBMP-2 has shown compatible biologic activity and enhancement of bone formation in a dose-dependent manner [6,9]. However, cyst-like bone void formation and soft tissue swelling have been reported as side effects [6]. Therefore, it is recommended to use ErhBMP-2 only for limited clinical cases, and at the lowest possible concentration [10-13].

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea, which is produced by drying the plant *Camellia sinensis* [14]. This biologically active catechin is considered to have many beneficial effects including anticancer and anti-inflammatory properties, as well as decreasing blood pressure and serum lipid levels without any significant side

effects [15,16]. EGCG has also been found to be effective for periodontal regeneration by inducing apoptotic cell death in osteoclasts [17,18]. In addition, EGCG also has direct effects on osteoblasts [19]. EGCG enhanced bone formation by inhibiting the synthesis of interleukin-6 and osteocalcin and by upregulating the synthesis of vascular endothelial growth factor [20-22]. In previous pre-clinical study, EGCG seemed to exhibit increase of bone density and volume in the cortical and cancellous bone[23]. No serious side effects of EGCG application have been found.

Biphasic calcium phosphate (BCP) bone substitute, a synthetic bone substitute, is composed of hydroxyapatite (HA) and β-tricalcium phosphate (TCP). BCP has a similar structure to that of human bone, and its resorption can be controlled by changing the ratio of HA to TCP [24,25]. BCP mainly has osteoconductive properties, but it does not have osteoinductive properties. Therefore, various efforts have been made to improve the osteogenic potential of BCP. Enhancement of osteogenic potential was confirmed when a biologically active peptide or ErhBMP-2 was coated on BCP using a specific coating procedure[26,27]. Even a low dose of ErhBMP-2 (0.05 mg/mL) led to improvements in osteogenic potential similar to those observed with a high dose (0.5 mg/mL) when a specific coating procedure was applied[26].

Based on the results of these studies, coating BCP with a combination of EGCG and ErhBMP-2 (0.05 mg/mL) could be considered as a way to improve osteogenic potential and minimize possible side effects. In a previous study in dogs, ErhBMP-2-/EGCG-coated BCP was confirmed to lead to

enhanced osteogenic potential [27]. The purpose of this study was to evaluate the enhancement of osteogenic potential by ErhBMP-2-/EGCG-coated BCP in a standardized rabbit sinus augmentation model.

II. MATERIAL AND METHODS

In vitro test

Alkaline phosphatase(ALP) activity assay

This assay was divided into three experimental groups and one control group. The experimental group consisted of three groups using EGCG [EGCG (Sigma-Aldlich, MO, USA) 5mg/ml] and ErhBMP-2[ErhBMP-2 (Cowellmedi, Busan, Korea) 0.05mg/ml] alone, and one group combining the two [ErhBMP-2 0.05mg/ml+EGCG 5mg/ml].

After 3, 7, and 14 days of incubation, osteoblasts were rinsed with phosphate-buffered saline (PBS; Invitrogen, CA, USA) solution, lysed by lysis buffer solution [25 mM Tris (pH 7.6), 150 mM NaCl, and 1% NP-40], and stored in an ice bath for 30 min. To measure alkaline phosphatase (ALP) activity, 50 μL of cell lysate was mixed with 200 μL of para-nitrophenylphosphate (Sigma-Aldlich), and the mixed solution was stored at 37° C for 30 min to activate the reaction. After 30 min, 50 μL of 3 N NaOH (Sigma-Aldrich) was added to stop the reaction. The absorbance of each solution was measured at 405 nm using an ELISA reader (SpectraMax 250, Thermo Electron Co, MA, USA). The rest of the cell lysate was used in measurements of the total protein content (Bradford protein assay kit, Sigma).

Alizarin red assay

The same group as the ALP assay was included. Extracellular matrix mineralization of osteoblasts was tested with alizarin red staining. After 14, 21, and 28 days of incubation, osteoblasts

were rinsed with PBS (Invitrogen) solution and fixed with 4% paraformaldehyde (Sigma-Aldrich) solution at room temperature for 20 minutes. After fixation, the cells were stained with 2% alizarin red (pH 4.2; Sigma-Aldrich) solution for 20 minutes and then washed twice with double-distilled water. The stained sample was reacted with 10% acetic acid (JT Baker, Center Valley, PA, USA) for 30 minutes and then stored at 85° C for 10 minutes. Supernatant was collected from the reacted solution after high-speed centrifugation (11,000 rpm for 15 minutes). The same volume of 10% ammonium hydroxide (Sigma-Aldrich) was added to the supernatant in order to neutralize it. The absorbance of each solution was measured at 405 nm using a microplate ELISA reader (SpectraMax 250, Thermo Electron Co.).

Preparation of ErhBMP-2-/EGCG-coated BCP

ErhBMP-2 (Cowellmedi) and EGCG (Sigma-Aldrich) immobilized grafting materials were stored at 70° C in a deep-freezer. When required, the frozen solution was placed in a lyophilizer (Ilshin Laboratory, Seoul, Korea) for 1 day to sublimate the liquid phase in a vial without denaturation of ErhBMP-2 and EGCG. The BCP surface (OSTEON, Dentium, Seoul, Korea) was coated with a combination of ErhBMP-2 and EGCG using a 3-step technique [23,24]. The first step was 3-aminopropyltriethoxysilane (Sigma-Aldrich), in which the OH- of HA was reacted with a silane coupling agent. The second step was to combine N-succinimidyl-3-maleimidopropionate (SMP; Sigma-Aldrich) with amino radicals. The last step was to combine EGCG and ErhBMP-2 with SMP. The success of the surface treatment was confirmed through field-emission scanning electron microscopy (FE-SEM;

S4800, Hitachi/Horiba, Japan) (Figure 1).

Cell viability test and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Human mesenchymal stem cells (hMSCs) were plated on each specimen(0.25 mL of BCP and 0.25 mL of ErhBMP-2-/EGCG-coated BCP), which had been previously prepared in a 24-well plate at a density of 1×10^4 cells/mL. The fluorescein diacetate(FDA; Sigma-Aldrich) staining technique was applied to count viable hMSCs that adhered to the specimen. At 2 and 24 hours after plating, hMSCs on the substrates were rinsed with PBS solution (Invitrogen) and incubated with FDA working solution (50 μg of FDA dissolved in 10 mL of PBS solution) for 30 seconds, and then washed 3 times using a PBS solution. The washed specimens were imaged with the aid of an inverted fluorescence microscope (CKX41, Olympus, Tokyo, Japan). An MTT assay was also applied. After the selected incubation periods, the samples were washed using a PBS solution and transferred to a new 24-well plate. MTT dye agent (1 mL, Sigma-Aldrich) was added to each well. After 3 hours of incubation in a 5% CO_2 incubator, 1 mL of isopropanol was added to each well and the plate was shaken for 30 minutes. The absorbance of each solution was measured using a microplate ELISA reader (SpectraMax 250, Thermo Electron Co.).

Surface analysis

The elemental composition, atomic concentrations, and chemical states of the elements present at each sample' s surface were measured quantitatively using X-ray photoelectron spectroscopy (XPS; K-Alpha ESKA, Thermo Electron Co.).

In vivo test

Study design and animals

Ten male New Zealand white rabbits (with 20 maxillary sinuses) weighing 2.5-3.0 kg were divided into 2 groups according to the type of bone graft materials used: ErhBMP-2-/EGCG-coated BCP(study group) on one side and BCP(control group) on the other side. Each group was then subdivided into 4- and 8-week healing groups before being euthanized. The selection process, management, surgical protocol, and preparation applied to the animal were reviewed and approved by the Institutional Animal Care and Use committee of Yonsei Medical Center, Seoul, Korea (2011-0056-4)(Table 1).

Surgical procedure

All surgical procedures were performed under general anesthesia. The animals were first anesthetized intramuscularly with a mixture of ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rompun, Bayer Korea, Seoul, Korea). The surgical site was isolated by shaving and sterilizing with povidone-iodine solution, and the animals were further anesthetized using 2% lidocaine with 1:100,000 epinephrine by infiltration. A straight incision was made in the sagittal plane, and a full-thickness flap was then reflected laterally to expose the maxillary sinus. Standardized and circular windows with a diameter of 6 mm were prepared bilaterally on the maxillary sinus using a saline-cooled trephine bur (3i Implant Innovation, Palm Beach Gardens, FL, USA)[27]. The reference point for micro computed tomography (micro-CT) analysis and specimen

preparation was indicated by a pin inserted at the point where the sagittal midline meets the imaginary line connecting the right and left windows. Following careful removal of the trephined bony disks, ErhBMP-2-/EGCG-coated BCP on one side (study group) and BCP on the other side (control group) were applied to the windows. The skin/periosteum flap was sultured using 4-0 Monosyn (glyconate absorbable monofilament, B-Braun, Aesculap, Center Valley, PA, USA), with periosteum being repositioned over the windows. Each animal was allowed a healing period of either 4 or 8 weeks postoperatively and then was euthanized.

Radiographic analysis: micro-CT

The block sections, including the augmented sinus and the surrounding bone were removed and then fixed in 10% neutral-buffered formalin for 10 days. X-ray imaging was then performed using micro-CT (SkyScan 1076, SkyScan, Aartselaar, Belgium) at a resolution of 35 μm (100 kV and 100 μA). OnDemand3D software (Cybermed, Seoul, Korea) was used to reconstruct the area of interest. The total augmented volume was measured volumetrically using the CT analyzer program supplied with the micro-CT system (SkyScan) as shown in Figure 2.[28] The total augmented volume and new bone volume were measured at the area of interest where the greatest volume was present in the reconstructed image.

Histologic analysis

After rinsing with water, all specimens were decalcified in 5% formic acid for 14 days and embedded in paraffin, followed by slicing into sections that were about 5 μm thick coronally along

the center of the augmented sinus. The two central-most sections were selected from each block, stained with hematoxylin and eosin, and examined under a light microscope (BX40, Olympus). Three regions of interest were selected from each slide: the defect margin area, the area near the Schneiderian membrane, and the center of the augmented area.

Histometric analysis

The following 2 parameters were measured using an automated image-analysis system (Image-Pro Plus, The Proven Solution, Chicago, IL, USA) [13,26]

New bone(%) = newly formed area/total augmented bone area X100

Remaining graft area(%) =remaining grafts area/total augmented bone area 100

Statistical analysis

Data from the ALP activity assay and alizarin red assay were analyzed statistically using 1-way analysis of variance, followed by Tukey's HSD multiple-range test as a *post-hoc* test (α = 0.05). The micro-CT data and histomorphometric measurements of the samples were analyzed statistically using standard statistical software (SPSS 15.0, SPSS, Chicago, IL, USA). The Mann-Whitney *U* test was used to compare differences between the control and study groups as well as differences within each group according to healing period. The cutoff for statistical significance was set at $P<0.05$, and the data are presented as mean and standard deviation values.

III. RESULTS

In vitro test

ALP activity assay

ALP activity increased in all groups during the experimental period, and it was greater in the combination group (ErhBMP-2/EGCG) than in the EGCG or control group at 3, 7, and 14 days (Figure 3).

Alizarin red assay

The concentration of alizarin red was higher in the ErhBMP-2 solutions than after 14 days of incubation. However, at 21 and 28 days there was not any significant difference between the ErhBMP-2 and combination (ErhBMP-2/EGCG) solutions, and the values found in the ErhBMP-2 and combination (ErhBMP-2/EGCG) solutions were higher than those in the EGCG and control solutions (P<0.05). (Figure 4).

Cell viability test: FDA staining and MTT assay

Adherent hMSCs became prominent in the ErhBMP-2-/EGCG-coated BCP at an early stage (within the initial 24 hours of incubation). The cell viability was not different between the BCP and ErhBMP-2-/EGCG-coated BCP. The results obtained in the MTT assay were consistent with FDA staining, with the level of cell proliferation being higher in the ErhBMP-2-/EGCG-coated BCP than in the control BCP (Figure 5).

Surface analysis

The binding energies were compared with those in the literature[29,30] to determine whether each reaction step had

been successfully completed. The Si2p spectrum near 100 eV that was evident in the XPS scan of BCP coated with ErhBMP-2 and EGCG indicated that the silanization of BCP was successful, since this spectrum was also found in the XPS scan of silane-treated BCP. Additionally, the presence of the N1s and C1s spectrums formed due to amide species indicated that the ErhBMP-2 and EGCG immobilization process was successful. Additional spectra, such as those of Na1s and Cl2p, obtained in the XPS scan of ErhBMP-2-/EGCG-coated BCP were due to the presence of PBS, which was used to preserve the coated specimens(Figure. 6). In the FE-SEM analysis, the BCP and ErhBMP-2-/EGCG-coated BCP surfaces showed different surface characteristics (Table 2.)(Figure. 7).

In vivo test

Radiographic and histometric analyses

Volumetric parameters were measured using the micro-CT data. The study and control groups exhibited similar mean new bone volume and total augmented volume after 4 and 8 weeks of healing. New bone volume and total augmented volume were greater in the study group than in the control group after 4 and 8 weeks of healing. The values after 8 weeks of healing were also slightly greater than those observed after 4 weeks of healing (Table 3). New bone (%) in the 4-week and 8-week study groups was greater than in the corresponding control groups ($P<0.05$). New bone (%) in the 8-week study group was also greater than in the 4-week study group ($P<0.05$) (Table 4).

Histologic analysis

The amount of vascularization and newly formed blood vessels in the 4-week control group (Figure 8D) was less than the 4-week study group (Figure 9D). The amount of immature woven bone, which was undergoing mineralization, was greater in the 4-week control group (Figure 8B) than in the 4-week study group (Figure 9B). Newly formed bone was sporadically found between the graft materials. Knife-edged bone graft materials were also identified in the 4-week study group (Figure 9B). However, a significant difference was not observed in this regard with the 4-week control group (Figure 8B).

In contrast, after 8 weeks of healing, the Schneiderian membrane had slightly thickened. The shape of the graft material had also become smoother, indicating the resorption of graft-material particles (Figure 10C and D). In addition, more stretching of the Schneiderian membrane, indicating that more new bone had formed toward the membrane, was found in this group (Figure 10C). In particular, a newly formed bony bridge was found after 8 weeks of healing between the graft material particles close to the Schneiderian membrane, showing very similar characteristics to the preexisting bone: an intensely stained, well-lined pattern of compact lamellar bone with a few osteocytes and marrow spaces (Figure 10C and D). The connective tissue seemed to have been replaced by mineralized tissue between 4 and 8 weeks.

IV. DISCUSSION

In the present study, a standardized rabbit sinus model with the same shape and size of the window was used [28]. The rationale behind this model was to provide the same conditions for the control and the study groups, thereby minimizing procedural errors. Additionally, the degree of anatomical variation between individuals is smaller in the rabbit sinus model than in larger animals, which allows a consistent reproduction of defects in different individuals. Preservation of the Schneiderian membrane was also possible with the delicate use of a trephine bur for osteotomy [28,31], as evidenced by the present histologic findings. In terms of group design, the 4-week healing group and 8-week healing group were selected to correspond to healing periods of 3-4 months and 6-8 months in human [32-34], since the metabolic rate of rabbits is roughly 3-4 times greater than that of humans.

The combination solution (ErhBMP-2/EGCG) and the ErhBMP-2 solution were equivalently effective in increasing ALP activity until 14 days. The combination solution (ErhBMP-2/EGCG) and the ErhBMP-2 solution were equivalently effective at increasing mineral nodule formation at 14, 21, and 28 days. As shown by the MTT assay and FDA staining, ErhBMP-2-/EGCG-coated BCP demonstrated a greater level of cell proliferation than BCP in the early stage. The cell viability observed with ErhBMP-2-/EGCG-coated BCP was equivalent to that observed with BCP. In summary, enhanced proliferation and differentiation of osteoblasts in response to ErhBMP-2/EGCG solution was confirmed. Cell viability was not influenced significantly by ErhBMP-2-/EGCG-coating. Based on the results of these *in*

vitro studies, which showed improved osteogenic potential and unchanged cell viability, ErhBMP-2-/EGCG-coated BCP can be considered as a possible alternative to BCP.

Surface analysis characterized the structural composition of ErhBMP-2-/EGCG-coated BCP via XPS. The presence of each element, distribution modality, and spectrum found in the XPS scan provided information on whether each reaction step had been successfully completed. By evaluating the spectra found on the XPS scan, it was confirmed that silanization and the ErhBMP-2 and EGCG immobilization process were successfully achieved. All specimens of ErhBMP-2-/EGCG-coated BCP were evaluated with this surface analysis before application in subsequent *in vivo* studies.

In the histomorphometric analysis, the 4-week and 8-week test groups showed more new bone (%) than the corresponding control groups ($P<0.05$), indicating that the osteogenic potential of BCP was improved by ErhBMP-2/EGCG coating. The 8-week test group showed more new bone (%) than the 4-week test group ($P<0.05$), confirming both the early and delayed effects of ErhBMP-2/EGCG coating. The overall enhancement of osteogenic potential in ErhBMP-2-/EGCG-coated BCP could support the possibility of replacing regular BCP with this coated BCP for clinical applications. Serious side effects of ErhBMP-2 were not found on the histologic evaluations. It seems that there was more soft tissue swelling in the study group, since the distance between particles in the study group was greater than in the control group in the histologic evaluation, and the total augmented volume was also slightly greater in the study group than in the control group in the radiographic evaluation.

However, this minor soft tissue swelling during the early healing period might have positively affected new bone formation in the test group; the space between particles in the 4-week study group was mainly composed of connective tissue, but it had changed into new bone in the 8-week study group. In addition, no serious inflammatory reaction was found in the study group. Therefore, the initial swelling in response to ErhBMP-2 could be considered as preparation for more new bone formation, rather than as a serious side effect.

In addition, delayed resorption of particles in response to the ErhBMP-2/EGCG coating was also confirmed; more remaining graft (%) in the histometric analysis and more remaining graft volume in the radiographic analysis were found in the study group than in the control group. Based on these results, it can be inferred that the EGCG coating may have induced apoptotic cell death of osteoclasts and inhibited their formation, as suggested by previous studies[18,32], thereby slowing the rate of bone resorption by osteoclasts. It is also anticipated that this less-resorptive property is particularly advantageous for the long-term stability of the newly formed bone against the air pressure within the sinus cavity, which may interfere with new bone formation through the activation of osteoclasts and bone resorption [32]. Consequently, it could affect the rate of bone formation and resorption during the healing phase. The replacement of bone graft material with newly formed bone does not necessarily occur in a 1:1 ratio, as new bone formation was clearly evident in both study groups [33-35]. Furthermore, when analyzing the correlation between the reduced amount of graft and the increased amount of new bone formation, both

study groups showed an increased amount of new bone formation that was relatively greater than the reduced amount of graft, suggesting that ErhBMP-2-/EGCG-coated BCP as a grafting material was more effective for new bone formation than BCP.

Our findings clearly suggest that ErhBMP-2-/EGCG-coated BCP could be a promising bone graft material, maximizing the osteogenic potential of ErhBMP-2, while simultaneously compensating for its side effects. In addition, owing to its less resorptive properties, it is especially advantageous in a situation where long-term new bone formation activity with enhanced osteogenic potential is required.

V. CONCLUSIONS

Within the limitations of this study, ErhBMP-2-/EGCG-coated BCP is effective as a bone graft material with enhanced osteogenic potential and minimized side effects.

VI. TABLES AND FIGURES

Table 1. Study design

Groups	Materials	Healing period	Number(%)
4 weeks control	BCP	4 weeks	5
8 weeks control	BCP	4 weeks	5
4 weeks study	ErhBMP-2-/EGCG-coated BCP	8 weeks	5
8 weeks study	ErhBMP-2-/EGCG-coated BCP	8 weeks	5

BCP: biphasic calcium phosphate,

ErhBMP-2: Escherichia coli-expressed recombinant human bone morphogenetic protein 2

EGCG: epigallocatechin-3-gallate

Table 2. Percentage of elemental contents(%)

Element	Silane-treated	All coated
Nitrogen	1.14	1.44
Silicon	2.03	1.69
Carbon	21.76	23.07

Table 3. Radiographic analysis in the standardized rabbit sinus model

Groups	New bone volume(mm^3)	Remaining graft volume(mm^3)	Total augmented volume(mm^3)
4-weeks control	25.05±3.52	33.23±2.85	58.28±5.98
4-weeks study	26.25±1.52	39.15±1.79	65.39±3.03
8-weeks control	30.63±8.44	38.60±10.18	69.23±18.00
8-weeks study	30.89±5.36	43.89±3.24	74.78±7.41

Table 4. Histometric analysis in the standardized rabbit sinus model

Groups	New bone	Remaining graft
4-weeks control	14.30±3.33	31.94±3.65
4-weeks study	23.34±4.81*	32.50±9.00
8-weeks control	19.71±10.54	27.00±5.87
8-weeks study	31.60±4.94*§	29.59±2.63

*: difference from control group in the same week $P<0.05$
§: difference from control group between 4 and 8 weeks in the same study group $P<0.05$

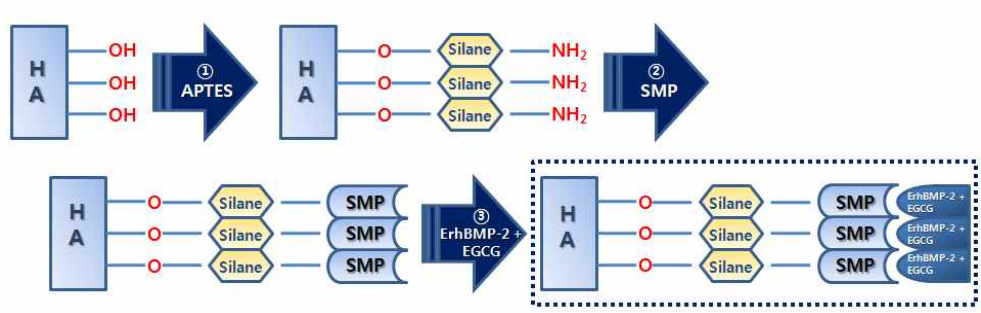

Figure 1.

Schematic diagram of preparation of BCP coated with low-concentration ErhBMP-2 and EGCG

1. APTES treatment

2. Bifunctional cross-linker (SMP) connection

3. Immobilized ErhBMP-2 and EGCG grafting

HA:hydroxyapatite, BCP:biphasic calcium phosphate

ErhBMP-2:Escherichia coli-expressed recombinant human bone morphogenetic protein2

EGCG:epigallocatechin-3-gallate

APTES:3-aminopropyltriethoxysilane

SMP: succinimidyl-3-maleimidopropionate

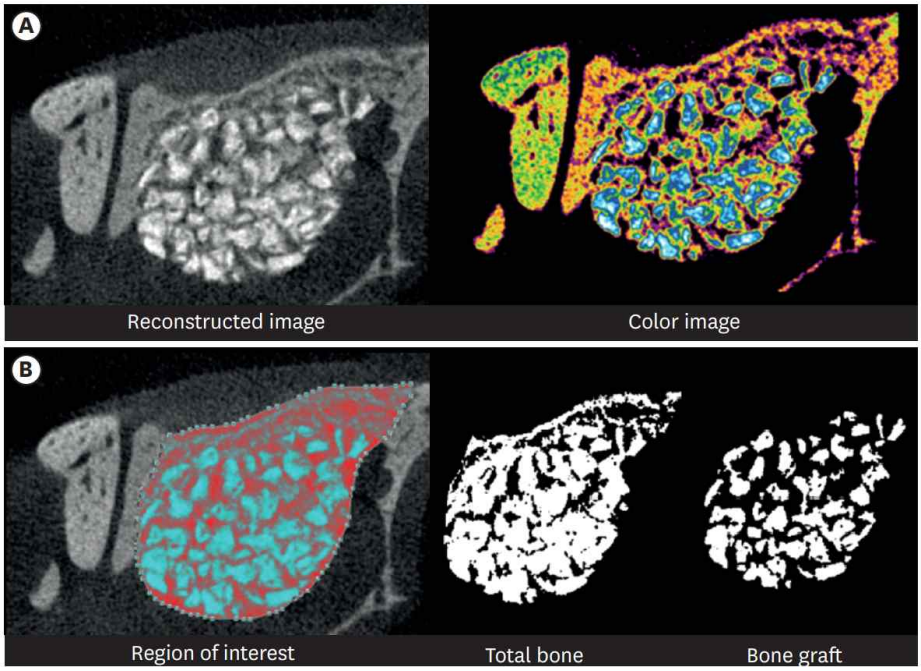

Figure 2. Radiographic analysis: micro-CT.

(A) Reconstructed image of micro-CT. (B) Region of interest: new bone volume was calculated as the remaining graft volume subtracted from the total augmented volume. CT: computed tomography

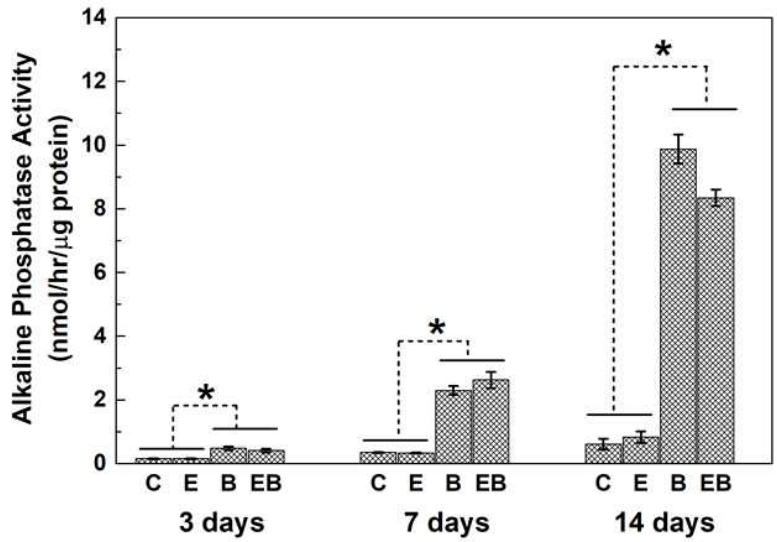

Figure 3. ALP activity assay

*: ALP activity was greater in the combination group (ErhBMP-2/EGCG) than in the EGCG or control group at 3, 7,14 days(P<0.05), C:control E: EGCG B:ErhBMP EB:EGCG+ErhBMP

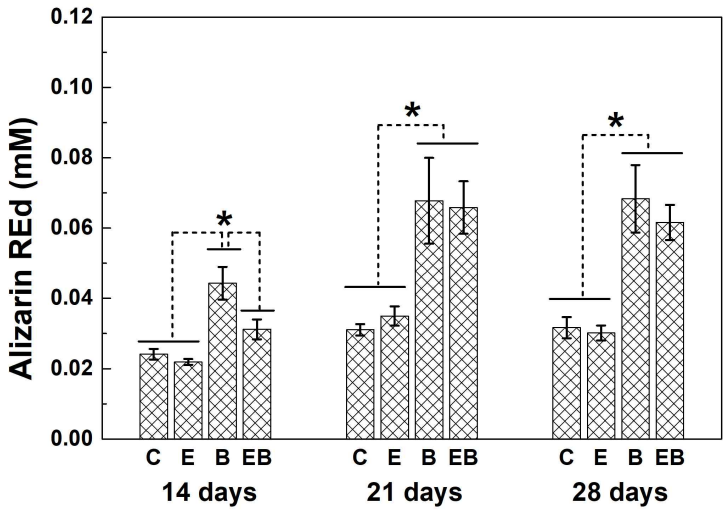

Figure 4. Alizarin red assay

The concentration of alizarin red was higher in the ErhBMP-2 group than in the other experimental groups after 14 days of incubation However, at 21 and 28 days there was no significant difference between the ErhBMP-2-only and combination (ErhBMP-2/EGCG) groups, and the values in both of these groups were higher than those in the EGCG and control groups ($P<0.05$). C:control E: EGCG B:ErhBMP EB:EGCG+ErhBMP

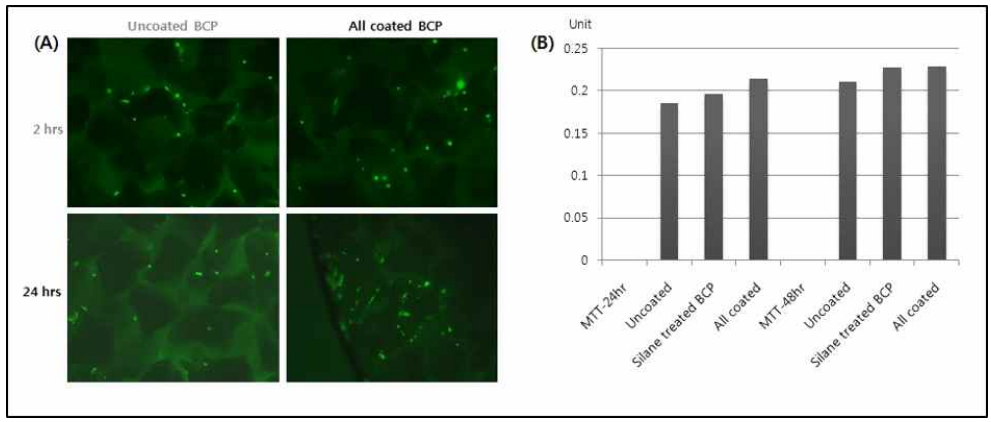

Figure 5. FDA staining and MTT assay

(A) Results of fluorescein diacetate staining at 2 and 24 hours. (B) Results of the MTT assay at 24 and 48 hours.

BCP: biphasic calcium phosphate

MTT:3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

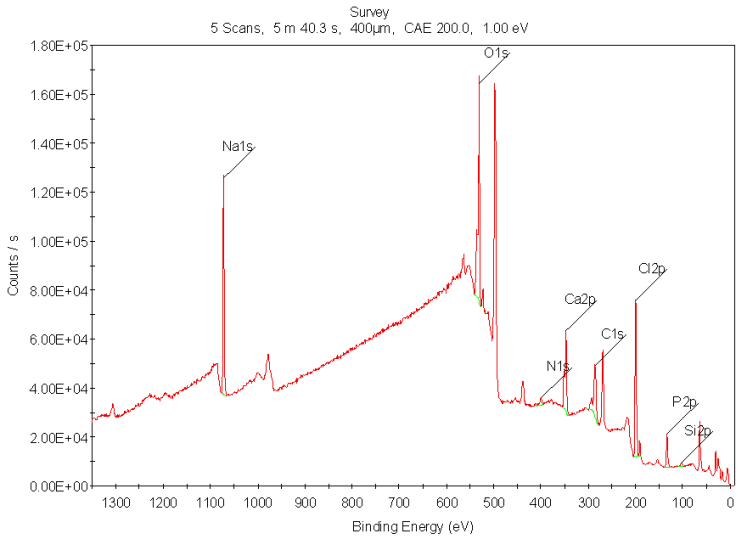

Figure 6. XPS analysis

The Si2p: Silanization of BCP
The N1s and C1s : The ErhBMP-2 and EGCG immobilization
The Na1s and Cl2p : The presence of PBS

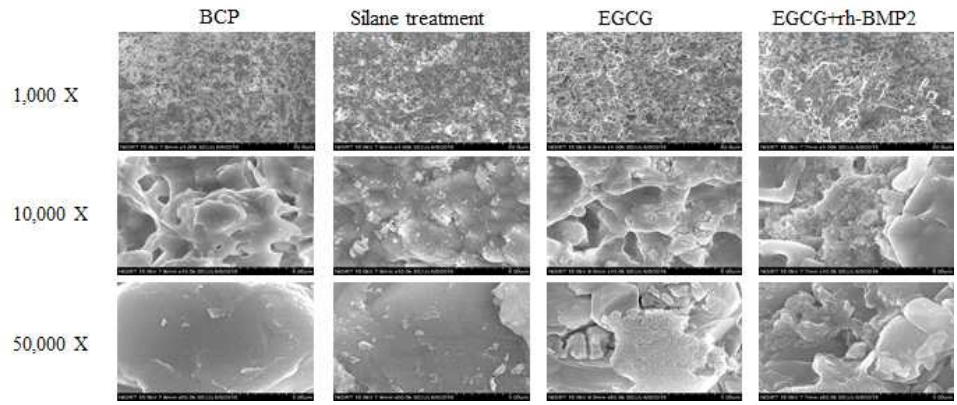

Figure 7. FE-SEM analysis

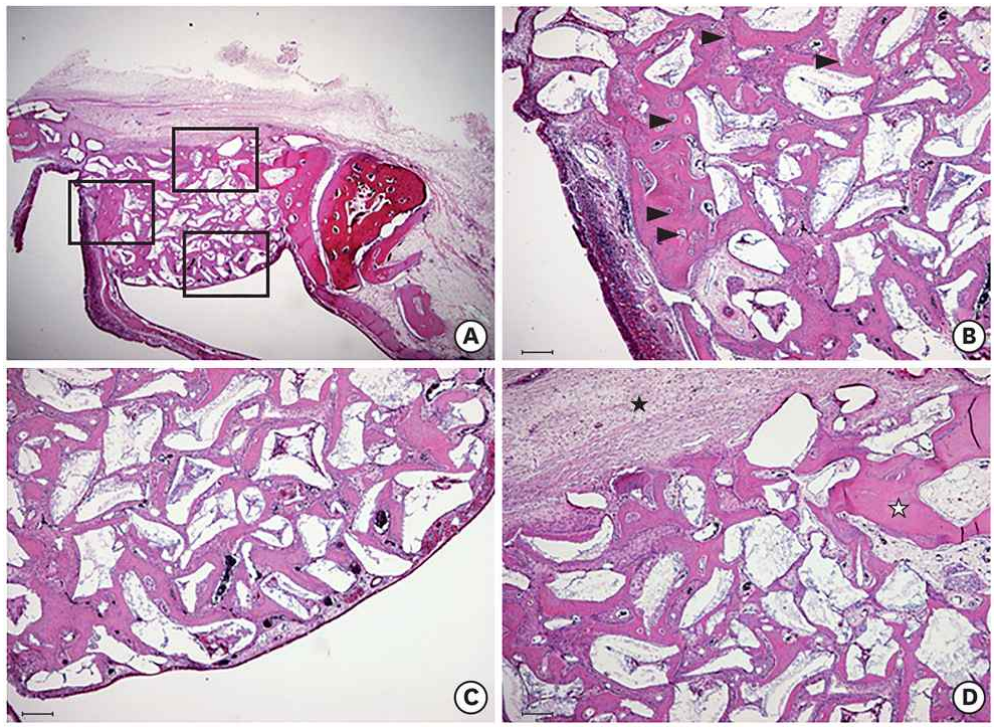

Figure 8. Histological findings of the 4-week control group.

(A) Area of interest (H&E). (B) Defect margin area (H&E, scale bar: 100 ㎛): immature osteocytes in the lacunae (arrowhead) were observed, which was a distinctive feature of the control group. (C) Near the Schneiderian membrane (H&E, scale bar: 100 ㎛). (D) Center of the augmented area (H&E, scale bar: 100 ㎛): the original curvature of the nasal bone was not restored, and loose connective tissue and adipose tissue were seen (black asterisk). Matured lamellar bone (white asterisk) can be hardly seen, either at the defect margin or in between graft materials. H&E: hematoxylin and eosin.

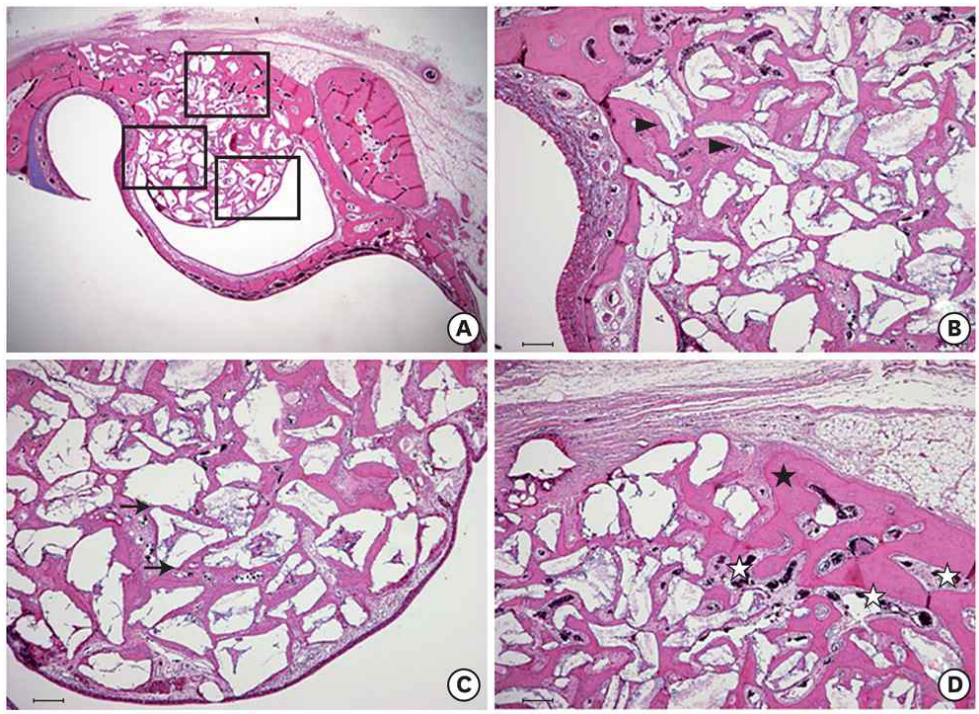

Figure 9. Histological findings of the 4-week study group

(A) Area of interest (H&E). (B) Defect margin area (H&E, scale bar: 100 ㎛): the number of immature osteocytes (arrowhead) was dramatically reduced. (C) Near the Schneiderian membrane (H&E, scale bar: 100 ㎛): black arrows indicate the shape of sharp, knife-edged bone graft materials, which were not greatly different from what was observed in the control group. (D) Center of the augmented area (H&E, scale bar: 100 ㎛): more vascularization and the presence of newly formed blood vessels (white asterisk) were identified. Black asterisks indicate that the majority of new bone formation occurred in a confined area, especially at the lateral side of the surgically created window. H&E: hematoxylin and eosin

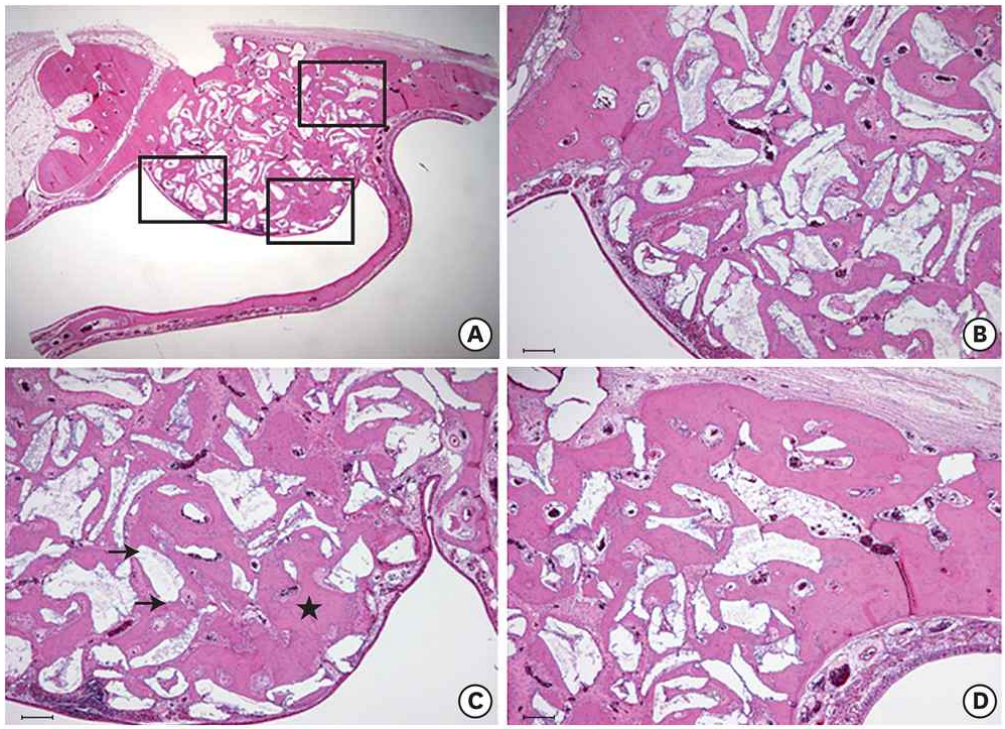

Figure 10. Histological findings of the 8-week study group

(A) Area of interest (H&E). (B) Defect margin area (H&E, scale bar: 100 ㎛). (C) Area near the Schneiderian membrane (H&E, scale bar: 100 ㎛): shallow, round, and ovoid-shaped graft materials were identified, indicating resorption of graft material particles. Intensely stained, well-lined patterns of compact lamellar bone (black asterisk) were prominent between graft material particles close to the Schneiderian membrane, showing very similar characteristics to the pre-existing bone. (D) Middle area (H&E, scale bar: 100 ㎛): surgically created window closure was markedly advanced and mature lamellar bone seemed to be predominant. H&E: hematoxylin and eosin.

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국문초록

BMP-2-/EGCG로 표면처리된 이상인산칼슘 골이식재의 골형성능 개선 평가

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연구목적: 연구의 목적은 대장균으로부터 유래한 재조합 인간 골형성 단백질-2 (Escherichia coli-derived recombinant human bone morphogenetic protein-2, ErhBMP-2)와 녹차 추출물 (epigallocatechin -3-gallate, EGCG)로 코팅한 이상인산칼슘 (biphasic calcium phosphate, BCP) 골 대체재의 골형성능 향상을 평가하는데 있다.

연구방법: EGCG, ErhBMP-2 , ErhBMP-2-/EGCG 용액내에서 조골세포의 분화와 세포외기질의 광화 정도를 각각 알칼리 인산 분해효소 (alkaline phosphatase, ALP) 활성 검사와 Alizarin red assay를 이용하여 확인하였다. ErhBMP-2-/EGCG로 코팅된 BCP의 표면을 X선 광전자 분광법 (X-ray photoelectron spectroscopy, XPS) 및 전계방사 주사 전자현미경 (field-emission scanning electron microscopy, FE-SEM)으로 분석하였다. 않은 BCP와 ErhBMP-2-/EGCG 코팅된 BCP에서 인간 중간엽 줄기세포의 세포 생존능을 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium

bromide (MTT) fluorescein diacetate (FDA) 염색을 통해 평가하였다. 뉴질랜드 백색 숫토끼 10마리의 양측 상악동에 표준화된 직경 6 mm의 골 결손부를 형성하였다. 골창을 제거하고 상악동막을 거상한 후에, ErhBMP-2-/EGCG로 코팅된 BCP를 실험군으로 한쪽 결손부에 적용하였다. 코팅되지 않은 BCP는 다른 결손부에 적용하여 대조군을 구성하였다. 수술 4주 후 또는 8주 후에 동물들을 희생하였고, 이식재와 주위조직을 생검하여 조직계측학적으로 분석하였다.

결과: ALP 활성 검사와 Alizarin red 검사에서는 대체로 ErhBMP-2와 ErhBMP-2/EGCG 용액에서 대조군과 EGCG용액보다 높은 값을 보였다 ($P<0.05$). ErhBMP-2-/EGCG로 코팅된 BCP의 표면을 XPS와 FE-SEM로 분석한 결과, 의도된대로 각 반응이 일어난 것을 확인할 수 있었다. MTT와 FDA 염색을 이용한 세포독성실험에서는 코팅되지 않은 BCP군과 ErhBMP-2-/EGCG를 코팅한 BCP군에서 차이가 없었다. 생체내 연구에서는 4주 후, 8주 후 실험군에서 각각의 대조군들에 비해 더 많은 신생골(%)이 관찰되었다 ($P<0.05$). 8주 후 실험군이 4주 후 실험군에 비해 더 많은 신생골(%)이 관찰되었다 ($P<0.05$).

결론: ErhBMP-2-/EGCG 코팅된 BCP는 효과적인 골 이식재로, 토끼의 상악동 골 증대술 모형에서 향상된 골형성능을 나타냈다.

주요어: 이상인산칼슘; 골형성단백질-2; 골대체재; 에피갈로카테킨-3-갈레이트
학번: 2016-31771