# 수산화인회석 캐리어에 코팅된 골형성단백 2형이 토끼 두개골 골재생 모델에서 인접한 조직에 미치는 영향

이재홍[1], 정정훈[1], 김유경[1], 이중석[1], 김영택[2], 방은경[3], 최성호[1]
연세대학교 치과대학 치주과학교실, 치주조직재생연구소[1],
국민건강보험공단 일산병원 치주과[2],
이화여자대학교 의학전문대학원 치주과학교실[3].

# Influence of BMP-2-loaded hydroxyapatite carrier on a neighboring ectopic site in the rabbit calvarial defect model

Jae-Hong Lee[1†], Chung-Hoon Chung[1†], You-Kyoung Kim[1], Jung-Seok Lee[1], Young-Taek Kim[2], Eun-Kyoung Pang[3], Seong-Ho Choi[1].
Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University[1], Department of Periodontology, National Health Insurance Service, Ilsan Hospital[2], Department of Periodontology, Graduated School of Medicine, Ewha Womans University[3].

† These authors contributed equally to this study.
Corresponding Author: Seong-Ho Choi, DDS, PhD.
Department of Periodontology, College of Dentistry, Yonsei University, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Korea
Tel: +82-2-2228-3189, Fax: +82-2-392-0398,
E-mail: shchoi726@yuhs.ac

## ABSTRACT

The aim of this study was to elucidate the influence of bone morphogenetic protein-2 (BMP-2)-loaded hydroxyapatite (HA) carrier on a neighboring surgical defect in the rabbit calvarial defect model. Two circular bicortical defects (8 mm in diameter) were created 2 mm apart on each rabbit calvarium ($n$=10 animals in each group). The rabbits were randomly divided into two groups: BMP-2-loaded HA and HA only. Control defects were designated as HAC (the sham surgical site located at the neighboring defect filled with HA only) and HAB (the sham surgical site located at the neighboring defect filled with BMP-2-loaded HA). Twenty animals were sacrificed at 2 and 8weeks postsurgery, and microcomputed tomographic, histologic, and histomorphometric evaluations were performed. The HAC and HAB groups showed small amounts of new bone formation at both healing periods. Radiographic, histologic, and histomorphometric features such as defect closure, new bone area, total augmented area, total bone/new bone volume, and closure volume did not differ significantly between the two groups. When evaluating the influence of BMP-2-loaded HA on a neighboring sham surgical site, inadvertent BMP-2 expression will not occur when the circular defects are separated by at least 2 mm.

Key words: Bone graft, Bone morphogenetic protein-2, Bone regeneration

## Ⅰ. Introduction

Bone morphogenetic protein-2 (BMP-2) regulates the proliferation, differentiation, and transformation of cells through autocrine and paracrine mechanisms, and induces bone regeneration[1]. The successful application of BMP-2, which is a promising bone regeneration factor, requires an appropriate carrier, but an ideal carrier system is yet to be found[2-4]. The rabbit calvarial defect model is very useful for evaluating the effects of BMP-2 in attempts to find an optimal carrier[5]. Several studies have created two to four circular defects with sizes from 6 to 15 mm (or even larger) to assess the bone regeneration ability of the growth factor of interest[6,7]. Although this design is a well-documented model, the ideal experimental model for assessing the effects of BMP-2 has not been clearly established.

The growth factor diffuses in the rabbit calvarial defect model not only within the local surgical site with grafting but also to the cells and tissue around the experimental defect to cause osteoblastic differentiation[8,9]. Therefore, unlike bone materials that exert an osteoconductive effect that is confined to the local defect, positive or negative control groups must be included when evaluating the effects on bone formation of growth factors such as BMP-2.

Another point to consider when assessing the influence of BMP-2 in the rabbit calvarium is the type of carrier material and the method used to load BMP-2. For example, changing the binding activity and release profile of BMP-2 will affect both the local and distant bone regeneration capacity[2,10]. Therefore, when investigating the effects of BMP-2 in accordance with the carrier, it is necessary to establish an experimental design in which the ectopic site will not be affected by BMP-2. The purpose of this study was therefore to verify whether inadvertent healing occurs at a neighboring sham surgical site that is 2mm from the experimental surgical site where BMP-2-loaded hydroxyapatite (HA) carrier is applied.

## Ⅱ. Material and methods

Preparation of recombinant human BMP-2- loaded HA

This study used HA (Bongros-HA, Seongnam, Korea) as a carrier of recombinant human BMP-2 (rhBMP-2; Cowellmedi, Seoul, Korea). The HA was composed of pure synthetic HA particles (80% porosity and 300 ㎛ pore size) with diameters of 0.6-1.0 mm. HA (0.1 g) was thoroughly impregnated in 0.15 ml of the rhBMP-2 for 10 minutes.

### Animals

Twenty male New Zealand white rabbits (mean body weight, 3.0- 3.5 kg) were used in this study. The animals were housed under standard laboratory conditions and fed with a standard pelleted laboratory diet. The selection, management, and preparation of the animals and the surgical procedures followed routine protocols approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea.

### Study design

Two circular bicortical defects (8 mm in diameter) were created 2 mm apart on each rabbit calvarium. The rabbits were randomly divided into two groups: rhBMP-2-loaded HA and HA only (with 10 animals in each group). In each rabbit, one of the defects was assigned to the negative control group and the other defect was assigned to either rhBMP-2-loaded HA or HA only. These defects were assigned to one of the following two control groups:

1. HAC group: the sham surgical site located at the neighboring defect was filled with HA only.
2. HAB group: the sham surgical site located at the neighboring defect filled with BMP-2-loaded HA.

Each rabbit was left to heal for either 2 weeks ($n$ = 10) or 8 weeks ($n$ = 10).

### Experimental protocol

To induce general anesthesia, the rabbits were injected intramuscularly with a mixture of ketamine hydrochloride (Ketalar VR, Yuhan, Seoul, Korea) and xylazine (Rumpun VR, Bayer Korea, Seoul, Korea). The skin was shaved and draped with povidone-iodine, followed by local anesthesia with 2% lidocaine (lidocaine-HCl, Huons, Seoul, Korea). The scalp was incised along the sagittal midline from frontal bone to occipital bone with a #15 surgical blade, and the calvarium was exposed. Under profuse saline irrigation, two circular bicortical holes were prepared using an 8 mm diameter trephine bur. The distance between the circular defects was set to 2 mm.

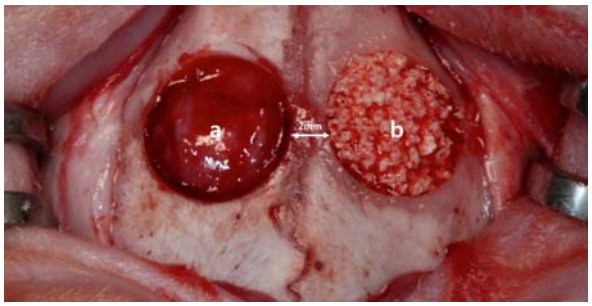


Fig. 1. Two circular defects (8 mm in diameter) were made in the rabbit calvarium separated by 2 mm (a) the sham surgical site located at the neighboring defect was filled with hydroxyapatite (HA) only (HAC) or recombinant human BMP-2 (rhBMP-2)-loaded HA (HAB), (b) the other surgical site randomly filled with graft materials (HA or rhBMP-2-loaded HA).

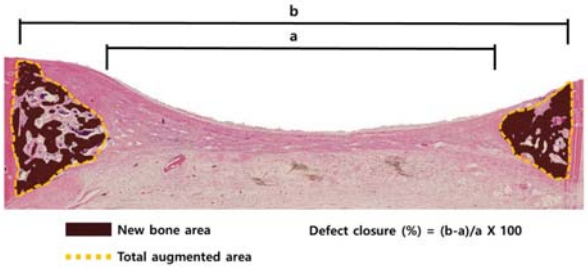


Fig. 2. Schematic diagram of the histomorphometric analysis. New bone area (NB, $mm^2$): the area of newly formed bone in the calvarial defect. Total augmented area (TA, $mm^2$): the area including newly formed bone, connective tissue, and vessels. Defect closure (DC, %): (distance between original defect margins) / (width of newly formed bone) ×100.

The randomly assigned graft material (rhBMP-2-loaded HA or HA only) was then grafted into one of the defects without using any barrier membrane (Fig. 1). The flap was sutured using 4-0 synthetic absorbable multifilament (VicrylPlus Antibacterial, Ethicon, New Jersey, USA), which was removed 10 days later. Twenty animals were sacrificed at 2 and 8 weeks postsurgery.

After harvesting bony blocks, all specimens were fixed in 10% buffered neutral formalin for 10 days. Dehydration was performed in 5% formic acid for 14 days. Specimens were embedded in paraffin, and then 5 ㎛ thick sections were cut along the sagittal direction of the rabbit calvarium. Each specimen was stained with hematoxylin and eosin.

## Radiographic analysis

Microcomputed tomographic scans (SkyScan 1173, Skyscan, Kontich, Belgium) were performed to obtain three-dimensional views of new bone in harvested sections. Digital images (in Digital Imaging Communication in Medicine format) were obtained at 130 kVp and 60 μA using 1.0 mm aluminum filtration. The following parameters were assessed:

1. Total bone volume (TV, $mm^3$) and new bone volume (NV, $mm^3$): newly formed bone volume.
2. Closure volume (CV, %): (total defect volume) / (newly formed bone volume)×100.

Since the bone graft materials were not applied to the sham surgical sites, TV and NV were considered as a single variable and calculated as such.

## Histologic and histomorphometric analyses

Histologic observations were made by a single examiner with the aid of a binocular microscope (Leica DM LB, Leica Microsystems, Wetzlar, Germany). After the microscopic examination, the defect closure(DF), new bone area(NB), and total augmented area(TA) histomorphometric parameters were measured using an automated image-analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) (Fig. 2):

1. DF (%): (distance between original defect margins) / (width of newly formed bone)×100.
2. NB ($mm^2$): newly formed bone area.
3. TA ($mm^2$): all tissue area including newly formed bone, connective tissue, adipose tissue, and vessels.

## Statistical analysis

All data were analyzed using standard statistical software (SPSS version 21.0, SPSS, Chicago, IL, USA). Descriptive data are presented as meanstandard deviation values and 95% confidence intervals. The independent *t*-test was used to assess differences between the study groups. The cutoff for statistical significance was defined as a *P* value of 0.05 or smaller.

## III. Results

### Clinical findings

All surgery sites were observed for any signs such as inflammation, allergic reactions, and exposure of materials after 2 and 8 weeks of healing. No rabbits showed any complications during the postoperative healing period.

### Microcomputed tomographic evaluation

Microcomputed tomographic analysis revealed that there was significantly more bone formation at 8 weeks than at 2 weeks in both the HAC and HAB groups. A clear demarcating line was evident between the 8- and 2-week groups. In addition, the minor formation of bony islands was visible on coronally sectioned images from the 8-week groups (Fig. 3, 4). After calculating TV/NV for

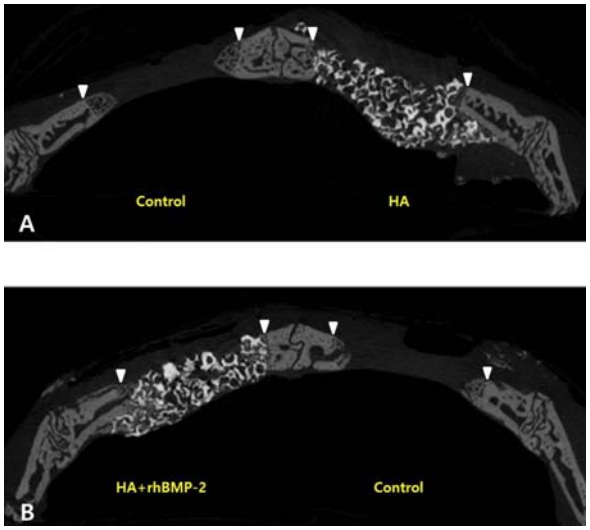


Fig. 3. Transversal microcomputed tomographic presentation at 2 weeks postoperatively: (A) the HAC control and (B) the HAB control. In both control-group defects, a small amount of new bone formation was observed and demarcating lines were clearly evident. arrowheads: original defect margins.

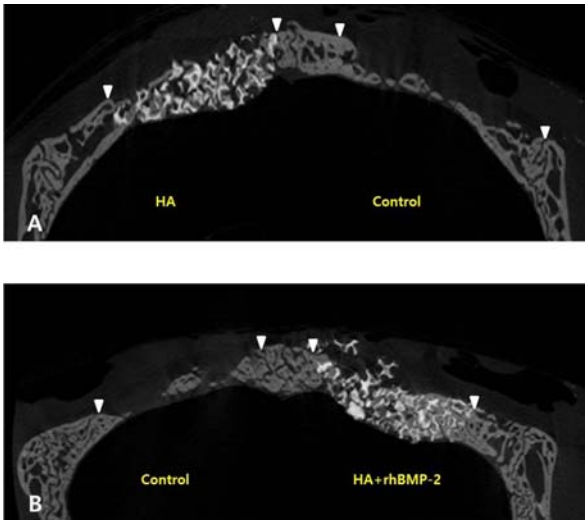


Fig. 4. Transversal microcomputed tomographic presentation at 8 weeks postoperatively: (A) the HAC control and (B) the HAB control. In both control-group defects, small amounts of new bone and bony island formation were observed. arrowheads : original defect margins.

Table 1. Microcomputed tomographic measurements at 2 and 8 weeks.

| | TV/NV ($mm^3$) | | CV (%) | |
|---|---|---|---|---|
| | 2 weeks | 8 weeks | 2 weeks | 8 weeks |
| HAC | 5.18±1.98 | 14.36±2.97[a)] | 34.50±2.45 | 51.88±8.92[a)] |
| HAB | 6.39±1.35 | 10.85±3.72[a)] | 33.38±1.31 | 51.94±7.63[a)] |

TV/NV: total bone and new bone volume, CV: closure volume, HAB: sham surgical site located at the neighboring defect filled with recombinant-human-BMP-2-loaded hydroxyapatite (HA), HAB: HAC: sham surgical site located at the neighboring defect filled with HA only.
There was no statistically significant difference between two groups at the same healing period.
[a)]Statistically significant difference compared to the 2-week group ($P < 0.05$).

Table 2. Histomorphometric measurements at 2 and 8 weeks.

| | DF (%) | | NB ($mm^2$) | | TA ($mm^2$) | |
|---|---|---|---|---|---|---|
| | 2 weeks | 8 weeks | 2 weeks | 8 weeks | 2 weeks | 8 weeks |
| HAC | 21.49±4.35 | 29.34±8.37[a)] | 1.35±0.35 | 1.43±0.47 | 6.15±0.99 | 6.94±0.96 |
| HAB | 20.87±9.15 | 32.14±2.83[a)] | 1.13±0.32 | 1.37±0.15 | 6.09±0.51 | 6.59±0.45 |

Data are mean ± standard deviation values.
DF: defect closure, NB: new bone area, TA: total augmented area.
There was no statistically significant difference between two groups at the same healing period.
[a)]Statistically significant difference compared to the 2-week group ($P < 0.05$).

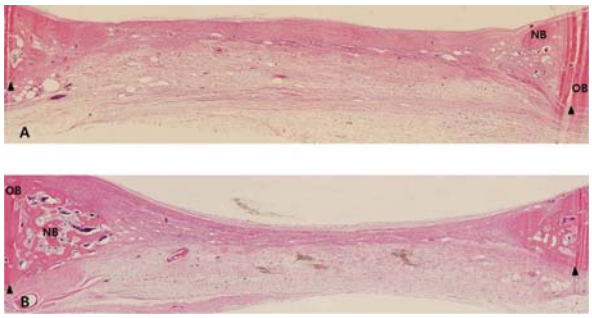


Fig. 5. Transversal histologic presentation at 2 weeks postoperatively: (A) the HAC control and (B) the HAB control. Specimens were composed mainly of loose connective tissue. A small amount of new bone formation was observed. (H&E, 40). arrowheads: original defect margins; OB: original bone.

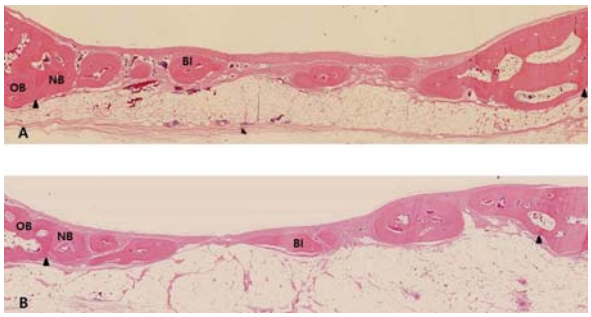


Fig. 6. Transversal histologic presentation at 8 weeks postoperatively: (A) the HAC control and (B) HAB control. Specimens were composed mainly of loose connective tissue. A small amount of new bone and bony islands were observed. (H&E, 40). arrowheads: original defect margins; BI: bone island.

HAC and HAB in both groups in three dimensions, the 8-week groups showed a statistically significant ($P < 0.05$) volumetric increase compared to the 2-week groups. TV/NV did not differ significantly between the HAC and HAB groups (Table 1).

### Histologic and histomorphometric evaluation

The histologic features were similar in the HAC and HAB groups. The defects had collapsed and were filled with loose connective tissue. The formation of new bone was greater in the 2-week groups than in the 8-week groups. However, there were no statistically significant differences, and some specimens exhibit a small amount of bony islands (Fig. 5, 6). In histomorphometric analysis, the DF ratio was significantly higher in the 8-week group (30.7%) than in the 2-week group (23.7%, $P < 0.05$). In both the HAC and HAB groups, NB and TA were greater in the 8-week groups than in the 2-week groups. However, this difference was not statistically significant, and even in the HAC and HAB groups there were no significant differences between DF, NB, and TA (Table 2).

## Ⅳ. Discussion

A previous study evaluated the influence of BMP-2-loaded biphasic calcium phosphate (BCP) carrier on a neighboring surgical defect in the rabbit calvarial defect mode[11)]. Those authors investigated the influence of BMP-2-loaded BCP (0.05 mg/mL BMP-2, HA: beta-tricalcium phosphate (β-TCP) = 3:7, 70% porosity and 250 ㎛ pore size), and reported that DF, NB, and TA

did not differ significantly between the defect containing BCP carrier loaded with BMP-2 and the neighboring control site grafted with BCP carrier only. The current study used HA as a carrier, and found that DF, NB, and TA were lower than in that previous study. However, as with the previous research, the present results indicate the absence of significant differences in bone formation between HAC and HAB.

HA, which is widely used as a BCP carrier, is a major component of bone that shows delayed resorption, unlike BCP containing β-TCP[12]. Accordingly, the use of an HA carrier system can induce bone regeneration with ample time, and a carrier that functions as a scaffold progressively changes into new bone[13,14]. In addition, due to the composition, structure, and affinity with BMP-2 differing between HA and BCP, the release profile, kinetics, and efficacy of BMP-2 are considered to be different[12,15].

The enzyme linked immunosorbent assay (ELISA) can be used to evaluate the affinity and release profile of the carrier[15,16], and is the most common method for analyzing the release kinetics of BMP-2 bound to a particular carrier. BMP-2-loaded BCP reportedly showed a burst release of 40.8% during the first 4 days[15]. In contrast, 12.1% of BMP-2 was released from HA during the first week, while after 4 weeks only approximately 14% had been released, thereby demonstrating a stagnant release profile. This is in line with previous authors concluding that BMP-2 exhibits a high affinity with HA and therefore does not result in sufficient release during the early healing phase[17,18].

A recent study that used a fluorescence-based retention assay to measure BMP-2-loaded HA observed an early burst release profile similar to measurement results from ELISA[12]. However, 42.9% of the BMP-2 was released during the first week, which was 3 times greater than the ELISA results. The total finally amount released was reported to be 93%. The results of an ALP assay that detects osteogenic differentiation caused by enzymes, and reflects bone induction, indicated a release profile similar to the release of BMP-2 from HA[12,15]. The results from an earlier study suggest that it is appropriate to evaluate the early healing at 2 weeks and late healing at 8weeks in the 8 mm circular defect model without grafting in the rabbit calvarium[7]. The results from the present study imply that it is reasonable to perform the assessments at 2 weeks, when the early burst release profile of BMP-2 from HA appears, and at 8 weeks, when the late healing phase occurs.

The binding activity with the carrier differs with the concentration of BMP-2. The effective dose also differs according to the species of experimental animal[10,19]. According to recent research in vitro, the binding capacity increased in a dose-dependent manner as the amount of BMP-2-loaded HA was increased up to 30 μg, while it remained constant for amounts greater than 30 μg[12]. The use of high concentrations of BMPs can cause side effects due to its accumulation in organs or inadvertent bone and adipose tissue formation[20-22]. Almost no cyst-like bone and adipose tissue in the specimens of the neighboring sham surgical site was observed in the present study, and so it may considered that BMP-2 had not affected the sham surgically created defect.

The development of effective carriers and BMP-2 to increase the mutual signaling of these cells is an area of intense research in tissue engineering[23]. The rabbit calvarial defect model is suitable for evaluating the effectiveness of carriers. Both previous and the present studies indicate that if the distance between the circular defects is 2 mm, the BMP-2-loaded carrier at the neighboring ectopic surgical site will not cause inadvertent BMP-2 expression. At the same time, it will be necessary to account for various factors when establishing an ideal experimental model in the rabbit calvarial defect, such as the concentration of BMP-2 and the temperature, which can affect the affinity of the carrier[12].

## Ⅴ. Conclusions

In summary, it was considered that a design with four circular defects separated by at least 2 mm without involving the sagittal line in the rabbit calvarial defect model is effective for assessing the effects of a BMP-2-loaded carrier.

## References

1. Olivares-Navarrete R, Hyzy SL, Haithcock DA, et al. Coordinated regulation of mesenchymal stem cell differentiation on microstructured titanium surfaces by endogenous bone morphogenetic proteins. Bone 2015;73:208-216
2. Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev

2003;55(12):1613-1629
3. Urist MR. Bone: formation by autoinduction. Science 1965;150(3698):893-899
4. Wozney JM, Rosen V, Celeste AJ, et al. Novel regulators of bone formation: molecular clones and activities. Science 1988;242(4885):1528-1534
5. Guda T, Darr A, Silliman DT, et al. Methods to analyze bone regenerative response to different rhBMP-2 doses in rabbit craniofacial defects. Tissue Eng Part C Methods 2014;20(9):749-760
6. Le Guehennec L, Goyenvalle E, Aguado E, et al. Small-animal models for testing macroporous ceramic bone substitutes. J Biomed Mater Res B Appl Biomater 2005;72(1):69-78
7. Sohn JY, Park JC, Um YJ, et al. Spontaneous healing capacity of rabbit cranial defects of various sizes. J Periodontal Implant Sci 2010;40(4):180-187
8. Ruschke K, Hiepen C, Becker J, Knaus P. BMPs are mediators in tissue crosstalk of the regenerating musculoskeletal system. Cell Tissue Res 2012;347 (3):521-544
9. Krebsbach PH, Gu K, Franceschi RT, Rutherford RB. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. Hum Gene Ther 2000;11(8):1201-1210
10. Choi Y, Lee JS, Kim YJ, et al. Recombinant human bone morphogenetic protein-2 stimulates the osteogenic potential of the Schneiderian membrane: a histometric analysis in rabbits. Tissue Eng Part A 2013;19(17-18):1994-2004
11. Lee JW, Lim HC, Lee EU, et al. Paracrine effect of the bone morphogeneticprotein-2 at the experimental site on healing of the adjacent control site: a study in the rabbit calvarial defect model. J Periodontal Implant Sci 2014;44(4):178-183
12. Kang W, Lee DS, Jang JH. Evaluation of Sustained BMP-2 Release Profiles Using a Novel Fluorescence-Based Retention Assay. Plos One 2015;10(4)
13. Tampieri A, Celotti G, Landi E. From biomimetic apatites to biologically inspired composites. Analytical and Bioanalytical Chemistry 2005;381(3):568-576
14. Hutmacher DW, Schantz JT, Lam CXF, et al. State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. Journal of Tissue Engineering and Regenerative Medicine 2007;1(4):245-260
15. Lee EU, Lim HC, Hong JY, et al. Bone regenerative efficacy of biphasic calcium phosphate collagen composite as a carrier of rhBMP-2. Clin Oral Implants Res 2015
16. Kim MS, Kwon JY, Lee JS, et al. Low-dose recombinant human bone morphogenetic protein-2 to enhance the osteogenic potential of the Schneiderian membrane in the early healing phase: in vitro and in vivo studies. J Oral Maxillofac Surg 2014; 72(8):1480-1494
17. Noshi T, Yoshikawa T, Dohi Y, et al. Recombinant human bone morphogenetic protein-2 potentiates the in vivo osteogenic ability of marrow/hydroxyapatite composites. Artif Organs 2001;25(3):201-208
18. Draenert FG, Nonnenmacher AL, Kammerer PW, et al. BMP-2 and bFGF release and in vitro effect on human osteoblasts after adsorption to bone grafts and biomaterials. Clin Oral Implants Res 2013;24(7): 750-757
19. Hong JY, Kim MS, Lim HC, et al. A high concentration of recombinant human bone morphogenetic protein-2 induces low-efficacy bone regeneration in sinus augmentation: a histomorphometric analysis in rabbits. Clin Oral Implants Res 2015
20. Park J, Ries J, Gelse K, et al. Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. Gene Ther 2003;10(13):1089-1098
21. Lee JS, Lee SK, Kim BS, et al. Controlled release of BMP-2 using a heparin-conjugated carrier system reduces in vivo adipose tissue formation. Journal of Biomedical Materials Research Part A 2015;103(2): 545-554
22. Zara JN, Siu RK, Zhang X, et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Eng Part A 2011;17(9-10):1389-1399
23. Guarino V, Causa F, Ambrosio L. Bioactive scaffolds for bone and ligament tissue. Expert Rev Med Devices 2007;4(3):405-418