Bone regenerative efficacy of biphasic calcium phosphate collagen composite as a carrier of rhBMP-2

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끝으로 이 자리를 빌어, 한결같은 마음으로 옆에서 있어주고 저를 늘 지지해주는 아내와 가족들에게 끊임없는 사랑과 무한한 감사를 말하고 싶습니다.

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이은웅

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Abstract

Bone regenerative efficacy of biphasic calcium phosphate collagen composite as a carrier of rhBMP-2

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Objective: This study compared the bone regenerative effects of a recombinant human bone morphogenetic protein 2 (rhBMP-2)-loaded collagen-based biphasic calcium phosphate composite (BCPC) and rhBMP-2-loaded biphasic calcium phosphate (BCP).

Materials and methods: The *in vitro* release profiles of rhBMP-2-loaded BCP and BCPC were measured. The animal surgery was performed on ten rabbits. Four 8-mm-diameter circular calvarial defects were made and filled with BCP, BCPC, rhBMP-2-loaded BCP (BMP + BCP) and rhBMP-2-loaded BCPC (BMP + BCPC). The animals were euthanized either 2 or 8 weeks after surgery.

Results: The initial burst release of rhBMP-2 was greater for BCP than for BCPC, and both presented a slow release pattern thereafter. In rabbit calvarial defects, the

space maintaining capability and graft resorption of all experimental groups did not show statistical differences at 2 and 8 weeks. New bone formation in the rhBMP-2-loaded groups was greater than in the non-loaded groups at both weeks, but the amount of new bone was comparable between both rhBMP-2-loaded groups at both weeks. There was a distinct histologic difference between the BMP + BCP and BMP + BCPC groups at 2 weeks; the new bone formation occurred more in the intergranular spaces and the BCP-to-bone contact was greater in the BMP + BCPC group, but these differences were no longer discernible at 8 weeks.

Conclusion: rhBMP-2 loaded BCP and BCPC significantly improved bone regeneration and BCPC led to a dense network of new bone and bone particles during the early healing period. BCPC can therefore be considered as a promising candidate for carrying rhBMP-2.

Keywords: biphasic calcium phosphate, bone morphogenetic protein 2, bone regeneration, bone substitutes, collagen

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I. Introduction

Bone morphogenetic protein (BMP) was discovered in the 1960s by Urist[1], since when its bone regenerative capacity has been well established[2-4]. However, appropriate delivery systems are essential to take full advantage of BMP, because continuous release and maintenance of BMP activity are difficult when it is applied alone[5, 6]. The optimal delivery system should release BMP constantly and for a sufficient time to induce new bone formation while maintaining space, so that defects can be adequately replaced by newly formed bone. In addition, the carrier system should be biocompatible, moldable, and degradable[7]. Various materials such as collagen[8], hydroxyapatite (HA)[9], polyethylene polymers[10], gels of autogenous,

allogenic, or alloplastic origin[11-13], and combinations thereof[14] have been investigated as candidate BMP carriers, but the ideal carrier has yet to be found.

Recent investigations of biphasic calcium phosphate (BCP) as a BMP carrier have produced promising results[15-18]. BCP itself, which comprises HA and β-tricalcium phosphate (β-TCP), has excellent biocompatibility and osteoconductivity as a graft material[19]. The micro- and macroporosity of BCP induce the attachment and proliferation of osteogenic cells[20], and the resorption rate of BCP can be controlled by varying the compositions of HA, which is more stable, and β-TCP, which is more soluble[21]. However, there are also limitations associated with using BCP as a BMP carrier. One problem with a BCP carrier is that insufficient BMP is released during the early healing phase because of its high affinity with HA[22]; BMP enhances early osteoblastic differentiation, which can compromise its effect on bone regeneration[3]. Another problem is that the performance of BCP loaded with BMP may vary with the defect morphology and the type of BCP used. Particle-type BCPs are susceptible to external compressive forces and can easily collapse when the defects are not contained[23], which can make their application technically demanding. In contrast, block-type BCP can be used for defects that require space to be maintained. However, several studies have demonstrated that block-type BMP-loaded BCP induces limited bone formation[23, 24]. Those studies tested BMP-loaded BCP blocks in a vertical augmentation experimental model and found that newly formed bone was restricted to the outer edges of the BCP block.

Collagen-based composite materials, which comprise a mixture of collagen

and calcium phosphate, could also be suitable carriers of BMP. It has been demonstrated that collagen-based composite materials can easily be loaded with BMP by soaking[25], and they subsequently release it slowly[26]. Moreover, these composite materials are easy to shape into forms suitable for various recipient beds, including to keep them in place compared with particle-type graft materials[27]. Thus, we hypothesized that a BCP–collagen composite (BCPC) could fulfill the requirement as a BMP carrier and also be convenient to use in clinical applications. However, BCPC has not been investigated extensively. This study compared the bone regenerative effects of recombinant human BMP-2 (rhBMP-2)-loaded BCPC and rhBMP-2-loaded BCP.

II. Materials and methods

Carrier preparation

Carriers

This study used BCP (OSTEON II, Genoss, Suwon, Korea) and BCPC (OSTEON II collagen, Genoss) as carriers of rhBMP-2. The BCP comprised HA and β-TCP at a ratio of 30 : 70 (particle size, 0.5–1.0 mm). The porosity was 70% and the macropore size was about 250 μm. The BCPC was a block-type graft material that uses collagen as a scaffold and was composed of BCP (OSTEON II, Genoss) and bovine type I collagen (4% wt), and had an average porosity of 70%. The BCPC used in this study was formed into a cylinder shape (diameter of 8 mm and height of 2 mm).

Loading rhBMP-2 onto the carriers

The Escherichia-coli-derived rhBMP-2 was produced at the research institute of Genoss in Suwon, Korea. We previously confirmed that 10 μg of rhBMP-2 induced a significant amount of new bone formation in rabbit[24]. For the purpose of determining the threshold dose, smaller dose was selected. The rhBMP-2 was reconstituted and diluted in buffer solution to a concentration of 50 μg/ml, and each of the graft materials (i.e., 0.1 ml of BCP and cylinder-form BCPC) was soaked in 0.1 ml of the prepared rhBMP-2. As a result, 5 μg of rhBMP-2 was loaded onto each scaffold.

In vitro study: rhBMP-2 release assay

The *in vitro* release of rhBMP-2 was measured over a period of 27 days, and the data were used to plot a cumulative release curve. In brief, a 50 μg/ml rhBMP-2 solution was prepared in phosphate-buffered saline (PBS), and 0.1 ml of rhBMP-2 solution was loaded onto each carrier. The final amount of rhBMP-2 loaded onto BCP and BCPC was 5 μg. Two samples per carrier were tested. The rhBMP-2 - loaded carriers were then placed into 24-well plates containing 1 ml of PBS (pH 7.4) and incubated at 37°C. The supernatants were collected and the tubes were replenished with fresh buffer at the following time points: 1, 2, 3, 4, 5, 6, 7, 10, 14, 20, and 27 days. The amount of rhBMP-2 in the supernatant at each time point was determined with an enzyme - linked immunosorbent assay (ELISA) kit (BMP-2 ELISA kit, Peprotech; Rocky Hill, NJ, USA) according to the manufacturer's instructions. The optical density of each well was determined at 450 nm using an absorbance microplate reader (PARADIGM; Beckman Coulter, Brea, CA, USA).

In vivo study

Animals

Ten male New Zealand white rabbits weighing 2.5–3.0 kg were used in this study. The animals were raised individually in separate cages under standard laboratory conditions and a standard diet. Animal selection, management, surgical

protocol, and preparation followed routine protocols approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea (approval number 2012-0031).

Study design

Four standardized circular defects with a diameter of 8 mm were made in the calvarium of each rabbit. These defects were randomly assigned to one of the following four experimental groups (Fig. 1):

1. BCP group - the defect was filled with BCP.
2. BCPC group - the defect was filled with BCPC.
3. BMP + BCP group - the defect was filled with rhBMP-2-loaded BCP.
4. BMP + BCPC group - the defect was filled with rhBMP-2-loaded BCPC.

Each animal was allowed to heal for either 2 weeks (n = 5) or 8 weeks (n = 5).

Surgical procedures

The animals were anesthetized with an intramuscular injection of a mixture of ketamine hydrochloride (Ketalar; Yuhan, Seoul, Korea) and xylazine (Rompun; Bayer Korea, Seoul, Korea). The surgical sites were shaved, wiped with alcohol and povidone iodine, and then locally anesthetized with 2% lidocaine. An incision was made along the sagittal midline from the frontal to the occipital bone, and a full-thickness flap was elevated to expose the calvarial bone. Four standardized circular bicortical calvarial defects were made using a trephine bur (outer diameter of 8 mm;

Genoss) under irrigation with cool saline. A calvarial bone core was then removed carefully and the dura mater was exposed. The defects were randomly assigned to one of the four experimental groups as listed above and filled as defined. All materials fitted snugly into the defect and did not protrude from it. The animals were killed at either 2 weeks (n = 5) or 8 weeks (n = 5) after surgery.

Histologic processing

Block sections of the defects were harvested, rinsed with sterile saline, and then fixed with 10% buffered formalin solution. They were decalcified with 5% formic acid and embedded in paraffin. Serial sectioning was performed at a thickness of 5 μm along the sagittal direction of the calvarial bone. Two sections close to the center were stained with Goldner's Masson trichrome and hematoxylin and eosin.

Analysis methods

Clinical observations

The defects were observed visually after 2 and 8 weeks of healing. The presence of inflammation, exposure of the graft material, and allergic reaction were evaluated.

Histologic and histomorphometric observations

For histologic observations, all of the specimens were examined using a

binocular microscope (Leica DM LB; Leica Microsystems, Wetzlar, Germany). Images of all specimens were captured and saved (cellSens Standard 1.11; Olympus Corporation, Center Valley, PA, USA). The total augmented area, new bone area, and graft materials were, respectively, color-coded with the aid of an image processing program (Photoshop CS6; Adobe Systems, San Jose, CA, USA) and measured using an automated image-analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). The following histomorphometric parameters were measured (Fig. 2):

1. Augmented area - the area of residual materials, bone, fibrovascular, adipose, and marrow tissues between the defect margins.

2. New bone area - the area of newly formed bone in the entire defect.

3. BCP-to-bone contact ratio - percentage of BCP particles in contact with newly formed bone.

4. Residual material area - the area of residual graft materials.

Statistical analysis

Statistical analyses were performed using SPSS software (version 21.0; SPSS, Chicago, IL, USA). The values of mean ± SD and median with range for each parameter were calculated. For assessing statistical significance among groups at each healing period, the Friedman test was used at a significance level of 0.05 and then, the Mann–Whitney tests including Bonferroni correction were used for post hoc pairwise

comparisons. Six pairwise tests were performed to compare four groups; thus, a conservative significance level of 0.00833 (0.05/6) was used. The Mann–Whitney test was used to analyze statistical differences between the 2- and 8-week healing periods at a significance level of 0.05.

III. Results

rhBMP-2 release assay

The release kinetics of rhBMP-2 from each of the carriers over 27 days was analyzed using an ELISA. More rhBMP-2 was released from BCP (2.04 μg) than from BCPC (0.48 μg) for the first 4 days, after which the two carriers released similarly small quantities of rhBMP-2. The total amounts of rhBMP-2 released from BCP and BCPC after 27 days were 2.37 and 0.62 μg, respectively, which were 47.27% and 12.40% of the originally loaded doses (Fig. 3).

Clinical observations

The wound-healing process was uneventful for all experimental sites during both healing periods. There was no clinical evidence of complications such as inflammation or exposure of graft material.

Histologic analysis

The 2-week healing period

Histologically, the healing after 2 weeks was similar in the BCP and BCPC groups. The collagen matrix of the BCPC was difficult to distinguish from the connective tissue observed in the intergranular spaces of the BCP group. A small amount of wedge-shaped new bone had formed, which was limited to the area of the defect margin. The space at the center of the defect was composed of loose connective tissue and graft materials, with no new bone formation (Fig. 4a,c). The amount of bone formation around the periphery of the defect margin was much greater in the BMP + BCP and BMP + BCPC groups than in the groups without rhBMP-2 (Fig. 4e,g).

High-magnification images of specimens from the BMP + BCPC group at the border of the newly formed bone revealed a different healing pattern compared to the other groups. In the BCP, BCPC, and BMP + BCP groups, osteoblasts and osteoids were incorporated within the graft particles, and intergranular spaces were filled mainly with loose connective tissue (Fig. 4b,d,f). In contrast, in the BMP + BCPC group, more osteoblasts and osteoids were found not only in close contact with the graft materials, but also in the intergranular spaces that had previously contained collagen matrix (Fig. 4h). In the center of the defect, specimens from all except the BMP + BCPC group were composed mainly of connective tissue and graft materials; newly formed bone was rarely evident. In contrast, new bone formation originating from the base of the defect was common in the BMP + BCPC group (Fig. 5).

The 8-week healing period

The size of the grafted BCP granules was similar in all of the groups after 8 weeks, and in all cases reduced during the healing period. The amount of new bone formation was greater at 8 weeks than at 2 weeks in both the BCP and the BCPC groups, but newly formed bone was mainly limited to the area of the defect margin. Bony islets were observed at the bottom of the defects in a few specimens. The defects were mostly filled with connective tissue and graft materials (Fig. 6a,c). In contrast, the defects in the BMP + BCP and BMP + BCPC groups were almost filled with newly formed bone and BCP granules. The amount of new bone in intergranular spaces in the BMP + BCP group was greater at 8 weeks than at 2 weeks, and the general histologic appearance was very similar in the BMP + BCP and BMP + BCPC groups. The superficial layer was infiltrated by loose connective tissue (Fig. 6e,g).

The newly formed bone in the BMP + BCP and BMP + BCPC groups presented with active remodeling and a more mature pattern (even in the center of the defect) compared to the BCP and BCPC groups. Numerous reversal lines and osteocytes were frequently evident. Osteoblasts lined the surface of some BCP particles, with osteoid tissue being observed. Few empty lacunae were found. Osteoblasts were observed on the surface of the particles in the BCP and BCPC groups, but fewer reversal lines were observed compared to the BMP-2-loaded groups (Fig. 6b,d,f,h).

In the center of the defect, the defect space in the BCP and BCPC groups was mostly filled with graft materials and connective tissue, with limited islands of new bone formation. In the BMP + BCP and BMP + BCPC groups, the intergranular spaces were almost totally filled with new bone (Fig. 7).

Histomorphometric analysis

The measured parameters are summarized in Tables 1–4. Due to the small number of experimental animals and the conservative significance level of 0.00833 from the Bonferroni correction, numerical *P* values for pairwise comparisons were presented.

The augmented area did not differ significantly among the experimental groups or between the 2- and 8-week healing periods (Table 1). The amount of new bone was more than twofold greater in the BMP + BCP ($P = 0.009$ with both the BMP and the BCPC groups) and BMP + BCPC groups ($P = 0.009$ with the BCP group and $P = 0.028$ with the BCPC group, respectively) than in the BCP and BCPC groups at 2 weeks. At 8 weeks, the difference in the amount of new bone became greater between rhBMP-2-loaded groups and non-loaded groups ($P = 0.009$). At both 2 and 8 weeks, the new bone area did not differ significantly between the BMP-loaded groups ($P = 0.602$ for 2 weeks and $P = 0.076$ for 8 weeks, respectively) (Table 2).

The BCP-to-bone contact ratio at 2 weeks was the highest in the BMP + BCPC group (57%), followed by the BMP + BCP group (38%). At 8 weeks, the contact ratios between the BMP + BCP and BMP + BCPC groups became comparable (84% and 87%, respectively). The contact ratios in the BCP and BCPC group were around 17–18% at 2 weeks, and 46% and 25% at 8 weeks, respectively (Table 3).

The collagen matrix of the BCPC group was not included in measurements of the residual material because it could not be distinguished from natural connective tissue. There was a statistically significant reduction in residual material between 2 and 8 weeks of healing in all groups ($P < 0.05$). The amount of residual material did not differ significantly among the groups at the same healing period (Table 4).

IV. Discussion

This study evaluated the efficacies of BCP and BCPC as carriers of rhBMP-2 using *in vitro* and *in vivo* models. The BCP used in this study comprised HA and β-TCP at a ratio of 30 : 70, because BCP with a high percentage of β-TCP is known to be effective at promoting new bone formation when used as a carrier of bioactive materials[28, 29]. Alam et al.[28] compared five rhBMP-2-loaded BCPs with different HA : β-TCP ratios, and found that bone formation was greatest when this ratio was 25 : 75. Similarly, Arinzeh et al.[29] reported that BCP with an HA : β-TCP ratio of 20 : 80 induced the greatest bone formation when accompanied by human mesenchymal stem cells.

The incorporation of collagen into BCP with a high percentage of β-TCP can provide additional advantages. As a carrier for BMP, collagen was demonstrated to induce new bone rapidly and compensate for the relatively slow bone-forming activity of BCP[30]. Instead, BCP can improve the poor durability of the collagen structure. Hence, combining collagen and calcium phosphate can increase the bone regenerative potential for carrying BMP while also maintaining space. From a clinical point of view, the moldability and adaptability of BCPC make it easier to shape and stabilize in various recipient beds[27], and blood and growth factors can be readily absorbed[25, 31].

In our *in vitro* study, both BCP and BCPC released part of rhBMP-2 (BCP,

47.27%; BCPC, 12.40%) for up to 27 days, and BCP showed a stronger initial burst pattern during the first 4 days. The initial loss of rhBMP-2 from BCP might be due to an incorporation failure, based on previous reports of a low release profile[6, 24, 32]. The release of rhBMP-2 from BCP may be related to the porous surface of BCP particles, which may mean that the release profiles of BCP vary with the surface topography. When BCP is combined with a collagen component, some of this collagen may overlay the porous surface so as to interfere with the release of rhBMP-2. This presumably affected the release of rhBMP-2 from BCP particles in BCPC, irrespective of whether or not it is due to incorporation failure. Conversely, the rhBMP-2 released from the collagen component can be reabsorbed onto the porous surface of BCP particles. The difference in the release profiles *in vitro* might be influenced by such interactions between BCP particles and the collagen component.

The *in vivo* situation for rhBMP-2 release involves complex interactions with a living body. After implantation, BCP particles and collagen in BCPC naturally undergoes a resorption process, while the presence of collagen components between BCP particles contribute the stabilization of blood clot[33]. Hence, rhBMP-2 from the collagen component and BCP particles can effectively give signals for osteoinduction during the early healing phase, which could have been reflected in the active new bone formation seen in the intergranular space of BCPC at 2 weeks in the present study.

When rhBMP-2 was not loaded onto BCP and BCPC, histologic differences were rarely observed. The collagen matrix of BCPC could not be distinguished from

natural connective tissue, and the size of the granules was similar for BCP and BCPC. In terms of new bone, both groups exhibited limited bone formation around the periphery of the defect margin. Similarly, bovine bone mineral and a collagen-bovine-bone mineral composite have been shown to exhibit similar histologic healing patterns[34].

The new bone formation was more homogenous and effective for BMP + BCPC than for BMP + BCP during the early healing period. In the BMP + BCPC group, osteoblasts and osteoids were found not only in close contact with BCP particles but also in the intergranular spaces. However, they were observed mainly at the border of BCP particles in the BMP + BCP group. Thus, the collagen component in the intergranular spaces might serve as a path for guiding the production of new bone in BCPC and provide space for bone regeneration during the early healing phase. Moreover, this could lead to a denser network of new bone and graft particles. The BCP-to-bone contact ratio was greater in the BMP + BCPC group than in the BMP + BCP group after 2 weeks of healing.

Unlike in the BMP + BCP group, newly formed bone was commonly observed in the middle of the defect in the BMP + BCPC group at 2 weeks. Although the middle of the defect was the most distant area from the native bone, its base, that is, dura mater is known to have osteogenic potential under appropriate stimulation[35]. It was demonstrated that the dura mater has abundant osteocomponent cells and pro-osteogenic cytokines[36]. The release profile of the BMP + BCPC group might provide adequate stimulation to the dura mater during the early healing period so that bone

formation is promoted in two directions: from native bone and the dura mater.

Even though there were some histologic differences between the BMP + BCP and the BMP + BCPC groups at 2 weeks, a release of the rhBMP-2 from BCP was similarly effective in new bone formation compared to BCPC. The amount of new bone did not differ significantly between the two groups at 2 weeks, and no histologic differences could be discerned at 8 weeks. Histologically, after 8 weeks, there was new bone with numerous reversal lines, and osteocytes filled in the intergranular spaces and formed close networks with BCP particles in both the BMP + BCP and the BMP + BCPC groups. In contrast, the bone-forming pattern remained immature in the BCP and BCPC groups.

With regard to the amount of rhBMP-2, several studies have shown that using BMP at levels higher than the threshold for bone formation does not increase the amount of new bone formation[17, 30, 37]. The prevalence and severity of complications of BMP, such as postoperative swelling, are known to be correlated with BMP overdosing[38, 39], which has prompted investigations into the minimum effective dose of BMP. The present study used 5 μg of rhBMP-2 per experimental site, which is smaller than the dose used in previous studies[15, 24], and greater amount of new bone formation was observed in the rhBMP-2-loaded groups in the present study.

The rabbit calvarial defect has been used for screening the effectiveness of various biomaterials. Although the critical-size defect - an orthotopic defect that will not heal without intervention - in the rabbit calvarium has been determined to be 10–15 mm[40-42], the 8-mm defect used in the present study has been considered a useful

defect model for evaluating bone regeneration[43, 44]. Moreover, this defect is close to being self-contained and is less affected by external pressure[45]. For vertical augmentation, the block type of BCPC may provide better mechanical stability than the particle type of BCP[23]. Hence, we ensured that all materials were applied to the defects snugly and did not protrude from it, which might have minimized the effects of the different types of BCP.

There is a logical concern that loading BMP-2 into one defect may affect an adjacent defect. The amount of new bone formed in the BMP-2-loaded groups was significantly greater than that in the non-loaded groups. Moreover, it was recently demonstrated that 5 μg of BMP would not affect the healing of an adjacent defect when two 8-mm-diameter defects were separated by a distance of 2 mm in the rabbit calvarium[46].

Within the limitations of this study, BCPC is a promising candidate as a carrier of rhBMP- 2. Incorporating collagen into BCP can provide several benefits such as a constant release profile, dense bone network during the early healing phase, and clinical manageability. The bone regenerative potential of BCPC-loaded BMP-2 in other types of defects should be investigated in future studies.

References

1. Urist, M.R. (1965) Bone: formation by autoinduction. *Science* 150: 893–899.
2. Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. & Wang, E.A. (1988) Novel regulators of bone formation: molecular clones and activities. *Science* 242: 1528–1534.
3. Hughes, F.J., Collyer, J., Stanfield, M. & Goodman, S.A. (1995) The effects of bone morphogenetic protein-2, -4, and -6 on differentiation of rat osteoblast cells in vitro. *Endocrinology* 136: 2671–2677.
4. Kim, C.S., Choi, S.H., Choi, B.K., Chai, J.K., Park, J.B., Kim, C.K. & Cho, K.S. (2002) The effect of recombinant human bone morphogenetic protein4 on the osteoblastic differentiation of mouse calvarial cells affected by Porphyromonas gingivalis. *Journal of Periodontology* 73: 1126–1132.
5. Winn, S.R., Uludag, H. & Hollinger, J.O. (1999) Carrier systems for bone morphogenetic proteins. *Clinical Orthopaedics and Related Research* 367 (Suppl.): S95–S106.
6. Geiger, M., Li, R.H. & Friess, W. (2003) Collagen sponges for bone regeneration with rhBMP-2. *Advanced Drug Delivery Reviews* 55: 1613–1629.
7. Uludag, H., Gao, T., Porter, T.J., Friess, W. & Wozney, J.M. (2001) Delivery systems for BMPs: factors contributing to protein retention at an application site. *The Journal of Bone and Joint Surgery*. Americal volume 83-A Suppl 1: S128–S135.
8. Choi, S.H., Kim, C.K., Cho, K.S., Huh, J.S., Sorensen, R.G., Wozney, J.M. & Wikesjo, U.M. (2002) Effect of recombinant human bone morphogenetic protein-2/absorbable collagen sponge (rhBMP-2/ACS) on healing in 3-wall intrabony defects in dogs. *Journal of Periodontology* 73: 63 – 72.

9. Rohanizadeh, R. & Chung, K. (2011) Hydroxyapatite as a carrier for bone morphogenetic protein. *Journal of Oral Implantology* 37: 659–672.
10. Isobe, M., Yamazaki, Y., Mori, M. & Amagasa, T. (1999) Bone regeneration produced in rat femur defects by polymer capsules containing recombinant human bone morphogenetic protein-2. *Journal of Oral and Maxillofacial Surgery* 57: 695– 698; discussion 699.
11. Viljanen, V.V., Lindholm, T.C., Gao, T.J. & Lindholm, T.S. (1997) Low dosage of native allogeneic bone morphogenetic protein in repair of sheep calvarial defects. *International Journal of Oral and Maxillofacial Surgery* 26: 389–393.
12. Santos, E.M., Radin, S., Shenker, B.J., Shapiro, I.M. & Ducheyne, P. (1998) Si-Ca-P xerogels and bone morphogenetic protein act synergistically on rat stromal marrow cell differentiation in vitro. *Journal of Biomedical Materials Research* 41: 87 −94.
13. Schwartz, Z., Somers, A., Mellonig, J.T., Carnes, D.L., Jr, Wozney, J.M., Dean, D.D., Cochran, D.L. & Boyan, B.D. (1998) Addition of human recombinant bone morphogenetic protein-2 to inactive commercial human demineralized freeze-dried bone allograft makes an effective composite bone inductive implant material. *Journal of Periodontology* 69: 1337–1345.
14. Alpaslan, C., Irie, K., Takahashi, K., Ohashi, N., Sakai, H., Nakajima, T. & Ozawa, H. (1996) Longterm evaluation of recombinant human bone morphogenetic protein-2 induced bone formation with a biologic and synthetic delivery system. *British Journal of Oral and Maxillofacial Surgery* 34: 414–418.
15. Miranda, D.A., Blumenthal, N.M., Sorensen, R.G., Wozney, J.M. & Wikesjo, U.M. (2005) Evaluation of recombinant human bone morphogenetic protein-2 on the repair of alveolar ridge defects in baboons. *Journal of Periodontology* 76: 210–220.

16. Jung, R.E., Weber, F.E., Thoma, D.S., Ehrbar, M., Cochran, D.L. & Hammerle, C.H. (2008) Bone morphogenetic protein-2 enhances bone formation when delivered by a synthetic matrix containing hydroxyapatite/tricalciumphosphate. *Clinical Oral Implants Research* 19: 188–195.
17. Jang, J.W., Yun, J.H., Lee, K.I., Jung, U.W., Kim, C.S., Choi, S.H. & Cho, K.S. (2012) Osteoinductive activity of biphasic calcium phosphate with different rhBMP-2 doses in rats. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology* 113: 480–487.
18. Wang, J., Zhang, H., Zhu, X., Fan, H., Fan, Y. & Zhang, X. (2013) Dynamic competitive adsorption of bone-related proteins on calcium phosphate ceramic particles with different phase composition and microstructure. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 101: 1069–1077.
19. Bohner, M. (2000) Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements. *Injury* 31(Suppl 4): 37–47.
20. Eggli, P.S., Muller, W. & Schenk, R.K. (1988) Porous hydroxyapatite and tricalcium phosphate cylinders with two different pore size ranges implanted in the cancellous bone of rabbits. A comparative histomorphometric and histologic study of bony ingrowth and implant substitution. *Clinical Orthopaedics and Related Research* 232: 127–138.
21. Nery, E.B., LeGeros, R.Z., Lynch, K.L. & Lee, K. (1992) Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/beta TCP in periodontal osseous defects. *Journal of Periodontology* 63: 729–735.
22. Noshi, T., Yoshikawa, T., Dohi, Y., Ikeuchi, M., Horiuchi, K., Ichijima, K., Sugimura, M., Yonemasu, K. & Ohgushi, H. (2001) Recombinant human bone morphogenetic protein-2 potentiates the in vivo osteogenic ability of marrow/hydroxyapatite composites. *Artificial Organs* 25: 201–208.

23. Kim, J.W., Choi, K.H., Yun, J.H., Jung, U.W., Kim, C.S., Choi, S.H. & Cho, K.S. (2011) Bone formation of block and particulated biphasic calcium phosphate lyophilized with Escherichia coliderived recombinant human bone morphogenetic protein 2 in rat calvarial defects. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiolofy, and Endodontology* 112: 298–306.

24. Kim, J.W., Jung, I.H., Lee, K.I., Jung, U.W., Kim, C.S., Choi, S.H., Cho, K.S. & Yun, J.H. (2012) Volumetric bone regenerative efficacy of biphasic calcium phosphate-collagen composite block loaded with rhBMP-2 in vertical bone augmentation model of a rabbit calvarium. *Journal of Biomedical Materials Research Part A* 100: 3304– 3313.

25. Friess, W., Uludag, H., Foskett, S., Biron, R. & Sargeant, C. (1999) Characterization of absorbable collagen sponges as recombinant human bone morphogenetic protein-2 carriers. *International Journal of Pharmaceutics* 185: 51 –60.

26. Geiger, M. (2001) Porous Collagen/Ceramic Composite Carriers for Bone Regeneration Using Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2). *Erlangen: Friedrich-AlexanderUniversity.*

27. Patino, M.G., Neiders, M.E., Andreana, S., Noble, B. & Cohen, R.E. (2002) Collagen as an implantable material in medicine and dentistry. *Journal of Oral Implantology* 28: 220–225.

28. Alam, I., Asahina, I., Ohmamiuda, K. & Enomoto, S. (2001) Comparative study of biphasic calcium phosphate ceramics impregnated with rhBMP-2 as bone substitutes. *Journal of Biomedical Materials Research* 54: 129–138.

29. Arinzeh, T.L., Tran, T., McAlary, J. & Daculsi, G. (2005) A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation. *Biomaterials* 26: 3631–3638.

30. Pang, E.K., Im, S.U., Kim, C.S., Choi, S.H., Chai, J.K., Kim, C.K., Han, S.B. & Cho, K.S. (2004) Effect of recombinant human bone morphogenetic protein-4 dose on bone formation in a rat calvarial defect model. *Journal of Periodontology* 75: 1364–1370.

31. Twardowski, T., Fertala, A., Orgel, J.P. & San Antonio, J.D. (2007) Type I collagen and collagen mimetics as angiogenesis promoting superpolymers. *Current Pharmaceutical Design* 13: 3608– 3621.

32. Autefage, H., Briand-Mesange, F., Cazalbou, S., Drouet, C., Fourmy, D., Goncalves, S., Salles, J.P., Combes, C., Swider, P. & Rey, C. (2009) Adsorption and release of BMP-2 on nanocrystalline apatite-coated and uncoated hydroxyapatite/betatricalcium phosphate porous ceramics. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 91: 706–715.

33. Kim, J.S., Cha, J.K., Cho, A.R., Kim, M.S., Lee, J.S., Hong, J.Y., Choi, S.H. & Jung, U.W. (2015) Acceleration of Bone Regeneration by BMP-2-Loaded Collagenated Biphasic Calcium Phosphate in Rabbit Sinus. *Clinical Implant Dentistry and Related Research*. 17: 1103-1113.

34. Sculean, A., Stavropoulos, A., Windisch, P., Keglevich, T., Karring, T. & Gera, I. (2004) Healing of human intrabony defects following regenerative periodontal therapy with a bovine-derived xenograft and guided tissue regeneration. *Clinical Oral Investigations* 8: 70 –74.

35. Levi, B., Nelson, E.R., Li, S., James, A.W., Hyun, J.S., Montoro, D.T., Lee, M., Glotzbach, J.P., Commons, G.W. & Longaker, M.T. (2011) Dura mater stimulates human adipose-derived stromal cells to undergo bone formation in mouse calvarial defects. *Stem Cells* 29: 1241–1255.

36. Greenwald, J.A., Mehrara, B.J., Spector, J.A., Chin, G.S., Steinbrech, D.S., Saadeh, P.B.,

Luchs, J.S., Paccione, M.F., Gittes, G.K. & Longaker, M.T. (2000) Biomolecular mechanisms of calvarial bone induction: immature versus mature dura mater. *Plastic and Reconstructive Surgery* 105: 1382–1392.

37. Lee, J.H., Kim, C.S., Choi, K.H., Jung, U.W., Yun, J.H., Choi, S.H. & Cho, K.S. (2010) The induction of bone formation in rat calvarial defects and subcutaneous tissues by recombinant human BMP-2, produced in Escherichia coli. *Biomaterials* 31: 3512–3519.
38. Smucker, J.D., Rhee, J.M., Singh, K., Yoon, S.T. & Heller, J.G. (2006) Increased swelling complications associated with off-label usage of rhBMP-2 in the anterior cervical spine. *Spine (Phila Pa 1976)* 31: 2813–2819.
39. Zara, J.N., Siu, R.K., Zhang, X., Shen, J., Ngo, R., Lee, M., Li, W., Chiang, M., Chung, J., Kwak, J., Wu, B.M., Ting, K. & Soo, C. (2011) High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Engineering Part A* 17: 1389–1399.
40. Shand, J.M., Heggie, A.A., Holmes, A.D. & Holmes, W. (2002) Allogeneic bone grafting of calvarial defects: an experimental study in the rabbit. *International Journal of Oral and Maxillofacial Surgery* 31: 525–531.
41. Xu, S., Lin, K., Wang, Z., Chang, J., Wang, L., Lu, J. & Ning, C. (2008) Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics. *Biomaterials* 29: 2588–2596.
42. Pripatnanont, P., Nuntanaranont, T. & Vongvatcharanon, S. (2009) Proportion of deproteinized bovine bone and autogenous bone affects bone formation in the treatment of calvarial defects in rabbits. *International Journal of Oral and Maxillofacial Surgery* 38: 356 –362.
43. Sohn, J.Y., Park, J.C., Um, Y.J., Jung, U.W., Kim, C.S., Cho, K.S. & Choi, S.H. (2010)

Spontaneous healing capacity of rabbit cranial defects of various sizes. *Journal of Periodontal & Implant Science* 40: 180–187.

44. Yang, C., Unursaikhan, O., Lee, J.S., Jung, U.W., Kim, C.S. & Choi, S.H. (2014) Osteoconductivity and biodegradation of synthetic bone substitutes with different tricalcium phosphate contents in rabbits. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 102: 80–88.
45. Yip, I., Ma, L., Mattheos, N., Dard, M. & Lang, N.P. (2015) Defect healing with various bone substitutes. Clinical Oral Implants Research. 26: 606-614.
46. Lee, J.W., Lim, H.C., Lee, E.U., Park, J.Y., Lee, J.S., Lee, D.W., Jung, U.W. & Choi, S.H. (2014) Paracrine effect of the bone morphogenetic protein-2 at the experimental site on healing of the adjacent control site: a study in the rabbit calvarial defect model. *Journal of Periodontal & Implant Science* 44: 178–183.

Figure Legends

Figure 1. Clinical photographs of the experiments. (a) Four 8-mm-diameter defects were made in the rabbit calvarium. (b) Each of the four defects was filled with different graft materials: (1) BCP, (2) BCP-collagen composite (BCPC), (3) rhBMP-2-loaded BCP (BMP + BCP), and (4) rhBMP-2-loaded BCPC (BMP + BCPC). Groups were allocated randomly.

Figure 2. Schematic diagram of the histomorphometric analysis. Augmented area = the area of residual materials, and bone, fibrovascular, adipose, and marrow tissues between the defect margins; New bone area = the area of newly formed bone in the entire defect; BCP-to-bone contact ratio = percentage of BCP granules in contact with newly formed bone; Residual material area = the area of residual graft materials.

Figure 3. Profiles of rhBMP-2 release from BCP and BCPC. More rhBMP-2 was released from BCP (2.04 μg) than from BCPC (0.48 μg) during the first 4 days, after which the two carriers released similarly small quantities of rhBMP-2. The total amounts of rhBMP-2 released from BCP and BCPC after 27 days were 2.37 and 0.62 μg, respectively, which were 47.27% and 12.40% of the originally loaded doses.

Figure 4. Transversal histologic sections in the BCP (a, b), BCPC (c, d), BMP +

BCP (e, f), and BMP + BCPC (g, h) groups after 2 weeks of healing. The amount of new bone was greater in the BMP + BCP and BMP + BCPC groups than in the BCP and BCPC groups. High-magnification images revealed osteoblasts and osteoids in close contact with the graft materials and in intergranular spaces in the BMP + BCPC group, but not in the intergranular spaces in the other groups. Arrowheads = defect margin; NB = new bone; G = graft material; C = connective tissue; OB = osteoblasts. Hematoxylin and eosin stain. (a, c, e, g) Original magnification: 40x; (b, d, f, h) original magnification: 200x.

Figure 5. Transversal histologic sections in the middle of the defect after 2 weeks of healing. Specimens from all except the BMP + BCPC group were composed mainly of connective tissue and graft materials; newly formed bone was rarely seen. In contrast, new bone formation originating from the base of the defect was found commonly in the BMP + BCPC group. (a) BCP, (b) BCPC, (c) BMP + BCP, (d) BMP + BCPC. Goldner's Masson trichrome stain, original magnification: 100x.

Figure 6. Transversal histologic sections in the BCP (a, b), BCPC (c, d), BMP + BCP (e, f), and BMP + BCPC (g, h) groups after 8 weeks of healing. In the BCP and BCPC groups, most of the newly formed bone was limited to the area of the defect margin. In contrast, in the BMP + BCP and BMP + BCPC groups, the defects were almost totally filled with newly formed bone and BCP granules. In the BMP + BCP and BMP + BCPC groups, numerous reversal lines and osteocytes were frequently

observed, and few empty lacunae were found. In the BCP and BCPC groups, osteoblasts were observed on the surface of the particles, but there were few reversal lines. Arrowheads = defect margin; RL = reversal line; OC = osteocyte. Hematoxylin and eosin stain. (a, c, e, g) Original magnification: 40x; (b, d, f, h) original magnification: 200x.

Figure 7. Transversal histologic sections in the middle of the defect after 8 weeks of healing. The BCP and BCPC groups were mostly filled with graft materials and connective tissue, with limited islands of new bone formation. In the BMP + BCP and BMP + BCPC groups, the intergranular spaces were almost totally filled with new bone. (a) BCP, (b) BCPC, (c) BMP + BCP, (d) BMP + BCPC. Goldner's Masson trichrome stain, original magnification: 100x.

Tables

Table 1. Augmented area (mm^2, group mean ± SD values, n = 5)

	BCP	BCPC	BMP+BCP	BMP+BCPC
2 weeks				
Mean ± SD	12.33 ± 2.04	13.84 ± 0.35	15.69 ±2.83	15.98 ± 1.85
Median	11.74	13.87	13.74	16.45
(Max., Min.)	(15.93, 10.96)	(14.20, 13.28)	(18.89, 13.40)	(18.07, 13.42)
8 weeks				
Mean ± SD	14.64 ± 1.33	13.31 ± 2.56	13.57 ± 2.35	15.14 ± 3.15
Median	14.64	12.83	13.51	15.20
(Max., Min.)	(16.36, 13.02)	(16.01, 10.13)	(16.57, 10.11)	(18.55, 10.09)

The Freidman test was used for statistical significance among groups at each healing time point ($P = 0.323$ for 2 weeks and $P = 0.668$ for 8 weeks, respectively).
The Mann–Whitney test was used to analyze statistical differences between the two healing time points. ($P > 0.05$ for all).

Table 2. New bone area (mm^2, group mean ± SD values, n = 5)

	BCP	BCPC	BMP+BCP	BMP+BCPC
2 weeks				
Mean ± SD	1.21 ± 0.46	1.29 ± 0.64	2.97 ± 0.35	2.76 ± 1.00
Median	1.33	1.21	2.77	2.26
(Max., Min.)	(1.63, 0.55)	(2.19, 0.44)	(3.37, 2.67)	(3.90, 1.88)
P-value (vs BCP)	-	0.917	0.009	0.009
P-value (vs BCPC)	0.917	-	0.009	0.028
P-value (vs BMP + BCP)	0.009	0.009	-	0.602
P-value (vs BMP + BCPC)	0.009	0.028	0.602	-
8 weeks				
Mean ± SD	2.66 ± 0.25*	2.16 ± 0.66	5.24 ± 0.76*	6.24 ± 0.24*
Median	2.66	2.37	5.24	6.24
(Max., Min.)	(3.00, 2.33)	(2.92, 1.15)	(6.47, 4.51)	(6.51, 5.86)
P-value (vs BCP)	-	0.175	0.009	0.009
P-value (vs BCPC)	0.175	-	0.009	0.009
P-value (vs BMP + BCP)	0.009	0.009	-	0.076
P-value (vs BMP + BCPC)	0.009	0.009	0.076	-

The Freidman test was used for statistical significance among groups at each healing time point ($P = 0.007$ for 2 weeks and $P = 0.003$ for 8 weeks, respectively).
Post hoc pairwise comparisons were conducted using the Mann–Whitney test including Bonferroni correction, and numerical *P*-values were presented.
The Mann–Whitney test was used to analyze statistical differences between the two healing time points (*$P < 0.05$).

Table 3. BCP-to-bone contact ratio (%, group mean ± SD values, n = 5)

	BCP	BCPC	BMP+BCP	BMP+BCPC
2 weeks				
Mean ± SD	17.44 ± 5.92	17.90 ± 11.01	38.05 ± 10.99	57.48 ± 8.54
Median (Max., Min.)	15.42 (21.83, 11.68)	12.54 (36.96, 9.90)	39.47 (49.70, 20.87)	58.49 (66.90, 44.32)
P-value (vs BCP)	-	0.602	0.016	0.009
P-value (vs BCPC)	0.602	-	0.028	0.009
P-value (vs BMP + BCP)	0.016	0.028	-	0.028
P-value (vs BMP + BCPC)	0.009	0.009	0.028	-
8 weeks				
Mean ± SD	46.88 ± 11.51*	25.56 ± 7.01	84.05 ± 15.34*	88.77 ± 2.95*
Median (Max., Min.)	44.20 (66.27, 35.71)	28.09 (29.86, 13.18)	90.12 (97.79, 66.49)	88.16 (93.18, 86.00)
P-value (vs BCP)	-	0.009	0.009	0.009
P-value (vs BCPC)	0.009	-	0.009	0.009
P-value (vs BMP + BCP)	0.009	0.009	-	0.754
P-value (vs BMP + BCPC)	0.009	0.009	0.754	-

The Freidman test was used for statistical significance among groups at each healing time point ($P = 0.004$ for both weeks).
Post hoc pairwise comparisons were conducted using the Mann–Whitney test including Bonferroni correction, and numerical *P*-values were presented.
The Mann–Whitney test was used to analyze statistical differences between the two healing time points (*$P < 0.05$).

Table 4. Residual material area (mm^2, group mean ± SD values, n = 5)

	BCP	BCPC	BMP+BCP	BMP+BCPC
2 weeks				
Mean ± SD	4.00 ± 1.04	4.45 ± 0.30	4.33 ± 0.75	4.51 ± 0.96
Median	3.62	4.51	3.97	3.96
(Max., Min.)	(5.77, 3.09)	(4.68, 3.95)	(5.32, 3.56)	(5.83, 3.68)
8 weeks				
Mean ± SD	46.15 ± 0.57*	2.16 ± 0.61*	2.22 ± 0.83*	2.25 ± 0.75*
Median	3.15	2.27	2.22	2.21
(Max., Min.)	(3.84, 2.57)	(2.83, 1.17)	(3.33, 1.25)	(3.16, 1.18)

The Freidman test was used for statistical significance among groups at each healing time point ($P = 0.323$ for 2 weeks and $P = 0.516$ for 8 weeks, respectively).
The Mann–Whitney test was used to analyze statistical differences between the two healing time points (*$P < 0.05$).

Figures

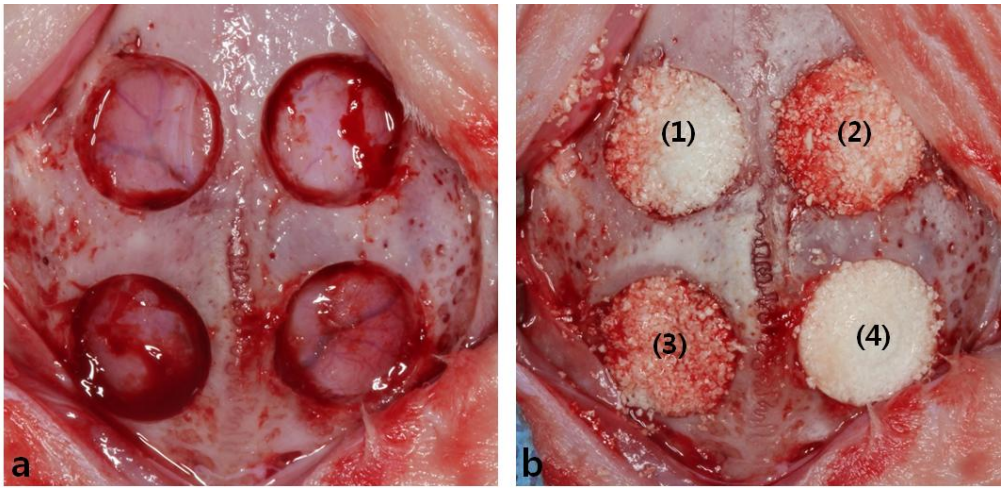

Figure 1

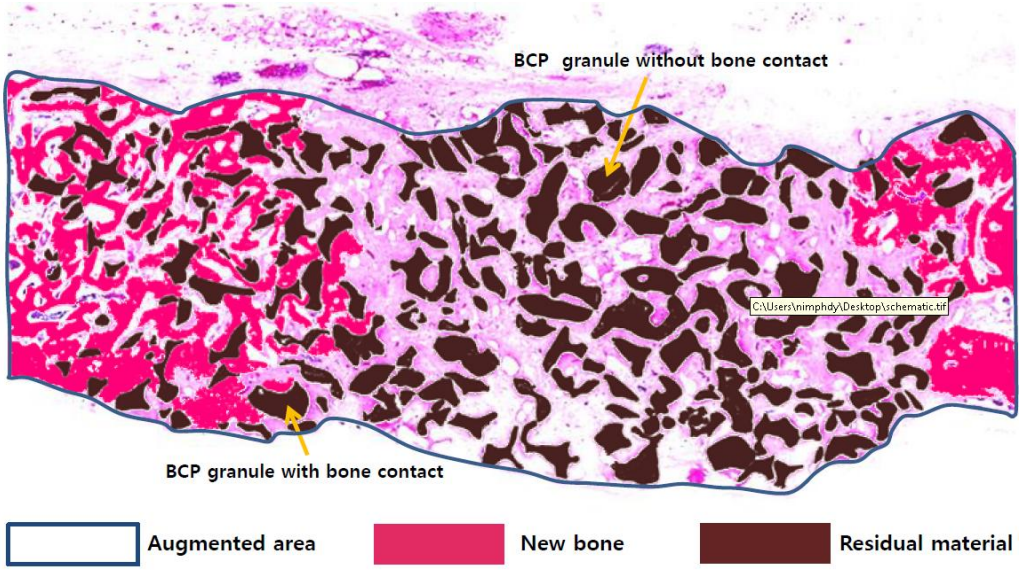

Figure 2

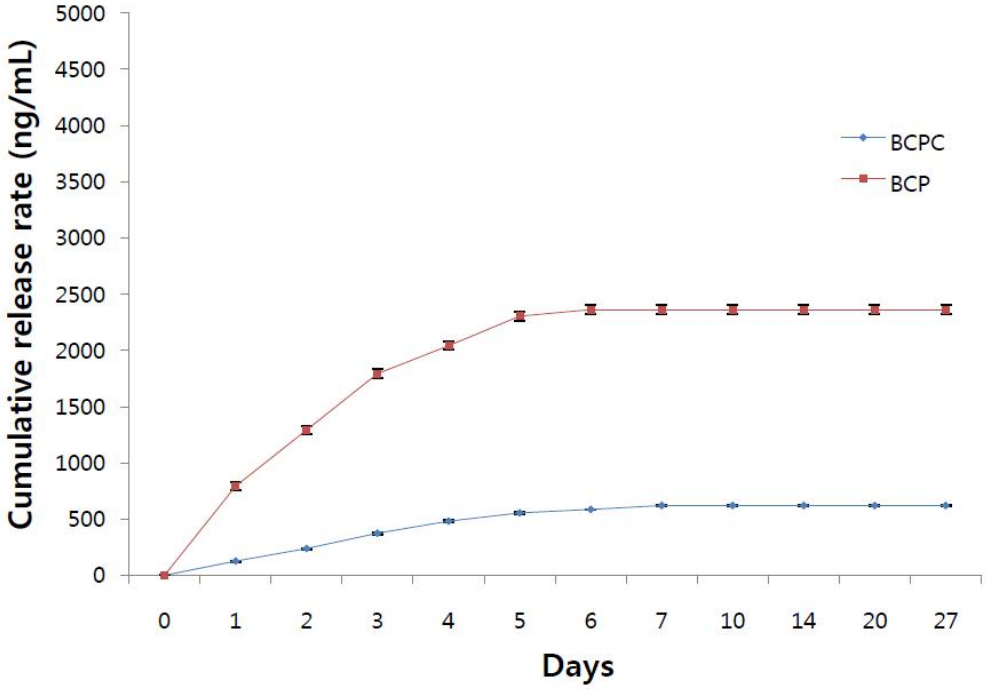

Figure 3

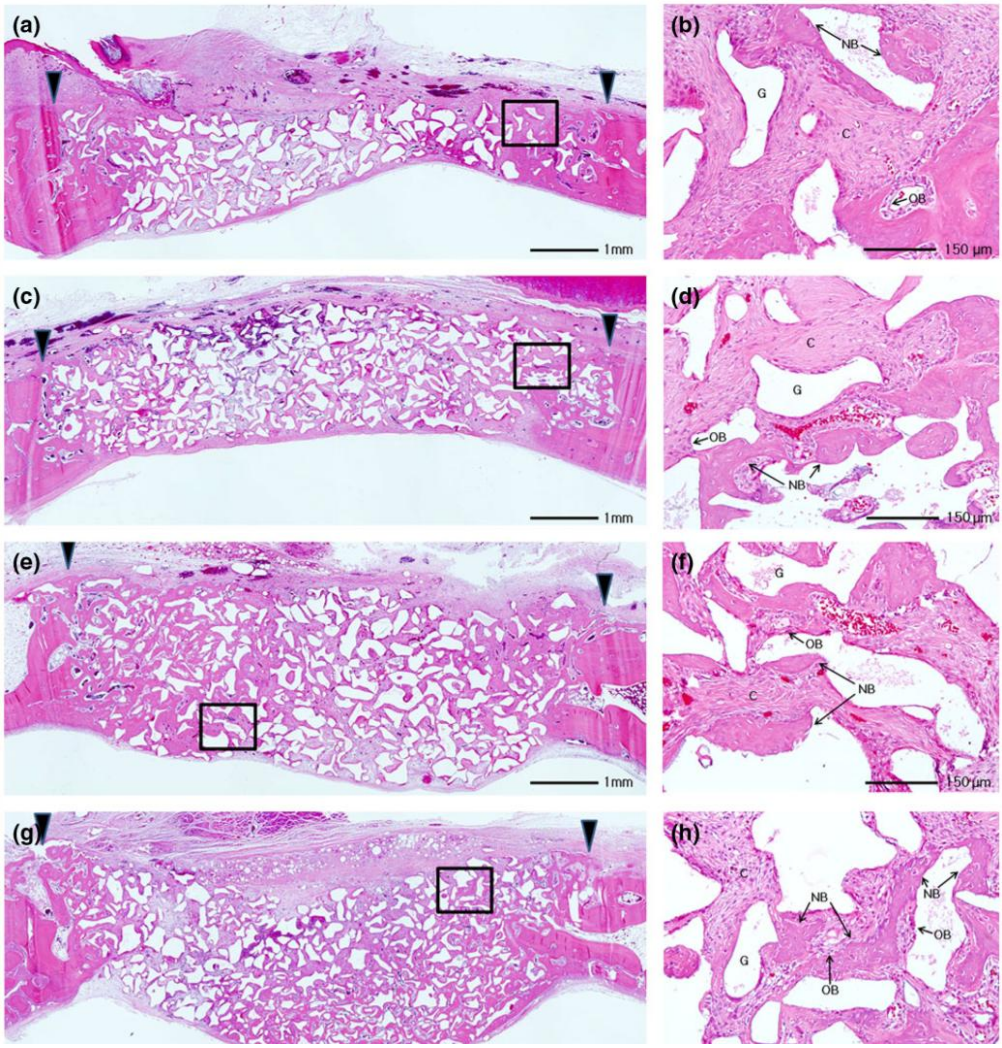

Figure 4

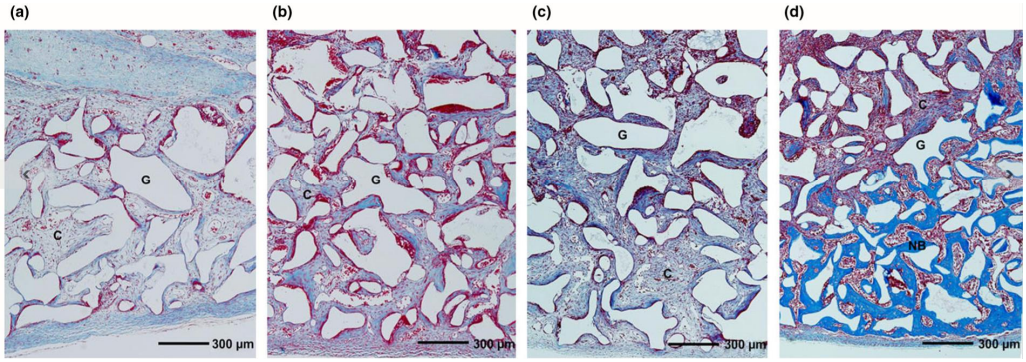

Figure 5

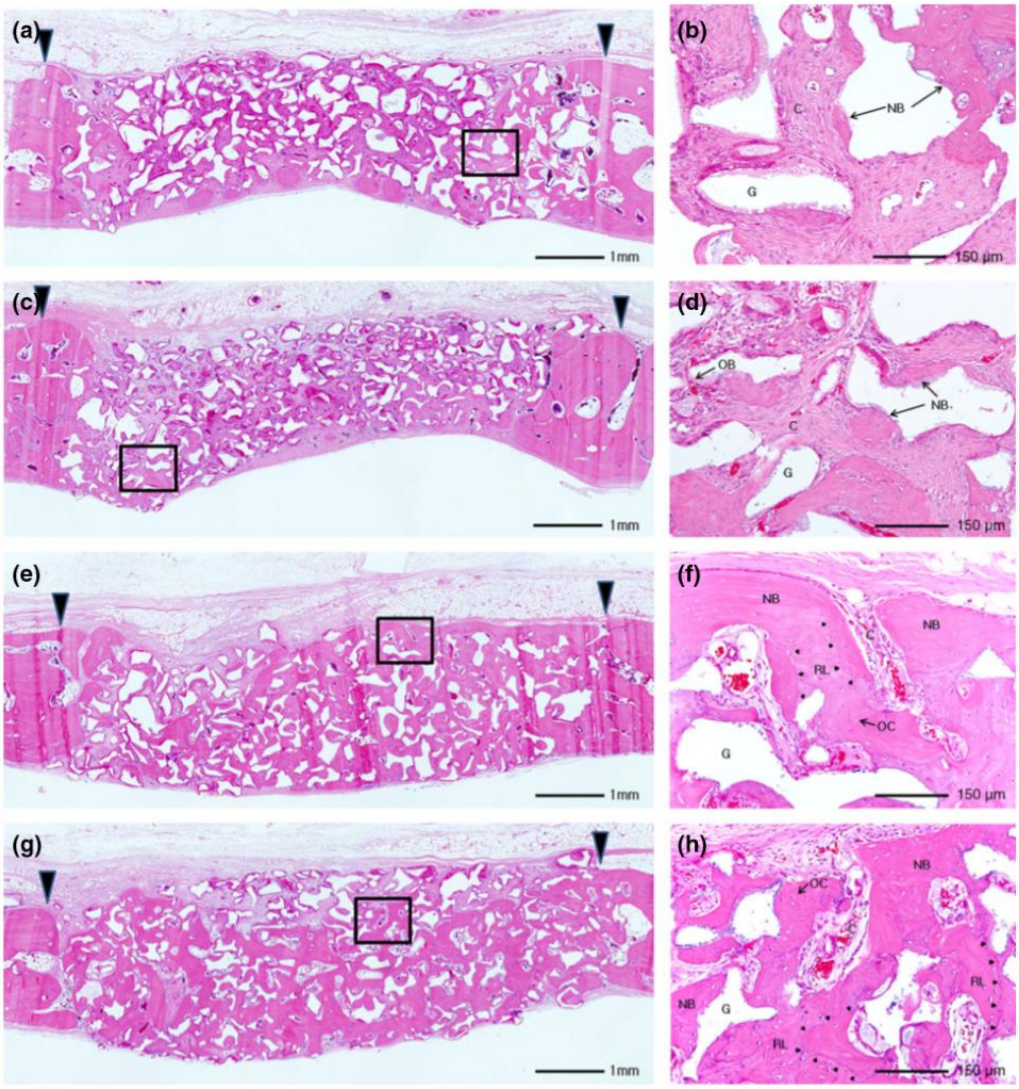

Figure 6

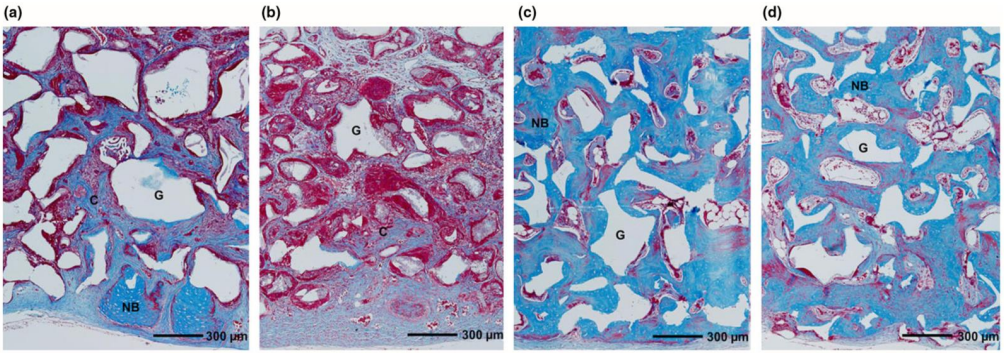

Figure 7

국문요약

골형성 단백질 운반체로서 이상인산칼슘 콜라겐 복합체의 골재생 효과

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이 은 웅

골형성 단백질의 우수한 골 재생능은 잘 알려져 있으나, 골형성 단백질을 효과적으로 전달할 수 있는 운반체는 개발되지 않은 상황이다. 골형성 단백질 운반체는 신생골 형성을 위하여 충분한 시간 동안 골형성 단백질을 방출하고 공간을 유지할 수 있어야 한다. 다양한 이식재중 이상인산칼슘 콜라겐 복합체는 생체적합성 및 공간유지 능력이 뛰어나고 골형성 단백질을 쉽게 흡수하는 특성이 있어 골형성 단백질의 운반체 후보로서 연구되어 왔다. 본 연구의 목적은 골형성 단백질 운반체로서의 이상인산칼슘과 이상인산칼슘 콜라겐 복합체의 골 재생능력을 비교, 평가하는 것이다.

생체 외 실험으로 5μg 의 골형성 단백질이 적용된 이상인산칼슘과 이상인산칼슘 콜라겐 복합체의 골형성 단백질 방출과정을 27 일간

관찰하였다. 생체 내 실험으로 총 10 마리의 백서를 대상으로 두개골에 4 개의 8mm 직경 원형 골 결손부를 형성하고, 각각의 결손부에 1) 이상인산칼슘 군 (BCP 군), 2) 이상인산칼슘 콜라겐 복합체 군 (BCPC 군), 3) 골형성 단백질 적용 이상인산칼슘 군 (BMP+BCP 군), 4) 골형성 단백질 적용 이상인산칼슘 콜라겐 복합체 군 (BMP+BCPC 군)을 임의로 적용하여 2 주, 8 주의 치유기간 후 조직학적, 조직계측학적으로 평가하였다.

생체 외 실험에서 처음 4 일간 이상인산칼슘이 이상인산칼슘 콜라겐 복합체와 비교하였을 때 더 많은 골형성 단백질을 방출하였으며, 그 이후로는 두 운반체 모두 유사한 양의 골형성 단백질을 방출하였다. 생체 내 실험에서 모든 군의 공간유지능력과 이식재의 흡수 정도는 차이가 없었다. 신생골 형성양은 두 치유기간에서 모두 골유도 단백질을 적용한 군이 골유도 단백질을 적용하지 않은 군보다 많았으며, 골유도 단백질을 적용한 두 군간에는 차이를 보이지 않았다. 2 주 치유기간에서 BMP+BCPC 군이 이외의 군과는 다른 조직학적 치유양상을 보였다. BMP+BCPC 군에서는 다른 군과는 달리 이상인산칼슘 이식재 사이의 공간에서 신생골이 형성되고 있었으며, 결손부의 중심부에서도 경막으로부터 신생골이 형성되고 있는 것이 관찰되었다. 또한 신생골과 인접해있는 이상인산칼슘의 비율도 더 큰 것으로 관찰되었다. 8 주의 치유기간이 경과한 후에는 BMP+BCP 군과 BMP+BCPC 군은 유사한 조직학적 치유양상을 보였다.

이상의 연구를 통해 이상인산칼슘과 이상인산칼슘 콜라겐 복합체 모두 골형성 단백질과 작용하여 골재생을 증가시킨 것을 확인하였다. 또한 이상인산칼슘 콜라겐 복합체는 초기 치유기간동안 신생골과 이식재간의 좀더 치밀한 연결을 형성하여 효과적인 골형성 단백질의 운반체임을 확인할 수 있었다.

핵심되는 말 : 이상인산칼슘, 골형성 단백질, 골 재생, 골 이식재, 콜라겐