# Recombinant Human BMP-2 Stimulates the Osteogenic Potential of the Schneiderian Membrane : A Histometric Analysis in Rabbits

Youna Choi

The Graduate School

Yonsei University

Department of Dental Science

# Recombinant Human BMP-2 Stimulates the Osteogenic Potential of the Schneiderian Membrane : A Histometric Analysis in Rabbits

Directed by Professor : Seong-Ho Choi

A Dissertation
Submitted to the Department of Dentistry
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Youna Choi

June 2014

This certifies that the Doctoral Dissertation of Youna Choi is approved.

Thesis Supervisor : Seong-Ho Choi

Kyoo-Sung Cho

Ui-Won Jung

Sung-Tae Kim

Young-Taek Kim

The Graduate School

Yonsei University

June 2014

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2014년 6월

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## Abstract

### Recombinant Human BMP-2 Stimulates the Osteogenic Potential of the Schneiderian Membrane : A Histometric Analysis in Rabbits

Youna Choi, M.S.D

Department of Dentistry
The Graduate School, Yonsei University

Directed by Professor Seong-Ho Choi, D.D.S., M.S.D., PhD.

This study evaluated the osteoinductive effect of recombinant human bone morphogenetic protein-2 (rhBMP-2)-coated biphasic calcium phosphate (BCP) carrier system on the grafted sinus area including surrounding tissues and the Schneiderian membrane.

Total 18 male rabbits were used in this study; two for *in vitro* and 16 for *in vivo* experiments. Schneiderian membranes taken from two animals were cultured with or without rhBMP-2, and quantitative reverse-transcriptase polymerase chain reaction analysis was performed. Both maxillary sinuses in

each of 16 animals were used to compare the *in vivo* effects of rhBMP-2-coated BCP (experimental group) and BCP alone (control group). In each animal, rhBMP-2-coated BCP was grafted into one of the maxillary sinuses, and the same amount of BCP alone was grafted into the contralateral site in random order. Radiologic and histometric analyses were performed at 2 and 8 weeks after surgery.

After two days of culturing with or without rhBMP-2, a significant increase in the expression of early osteoblast (*RUNX2*, Type I collagen, Alkaline phosphatase, Osteopontin*)* could be observed. Different histologic healing patterns were observed after 2 weeks in experimental and control sites: newly formed bone lining the reflected sinus membrane without bone formation was observed at the central areas of experimental sites (window = 0.06%; center = 0%; membrane = 20.86% of new bone), whereas evenly distributed new bone formation was observed at the control sites (window = 7.27%; center = 7.41%; membrane = 15.58% of new bone).The augmented volume was well maintained at both the experimental and control sites during the experimental period, but at 2 weeks the augmented volume was greater at the experimental sites than at the control sites (232.62 $mm^3$ and 195.29 $mm^3$, respectively; $p < 0.001$).

These results suggest that good space maintenance in sinus augmentation

is achieved with BCP, while the osteoinductive potential of the sinus membrane is activated at the early stage of healing with rhBMP-2.

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**Keywords :** rhBMP-2, biphasic calcium phosphate, Schneiderian membrane, bone regeneration, animal model

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Youna Choi, M.S.D

Department of Dentistry
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## I. Introduction

Quantitatively and qualitatively successful vertical augmentation for dental implants has been achieved in severely resorbed posterior maxillae using sinus floor elevation combined with bone grafting.[1-3] Several systematic reviews have confirmed predictable long-term clinical results for maxillary sinus grafts, which could be attributed to the development of superior bone substitutes, rough-surfaced implants, and improved surgical skills.[2]

Nevertheless, the requirement of an extended healing time to gain adequate levels of bone quality remains a drawback of this procedure.[4-6] Dental implants in the grafted maxillary sinus should be supported largely by the regenerated bone in the augmented area, and so the quality of regeneration in all grafted sinuses may be a major factor for successful results. However, the quality of regenerated bone in grafted sinus can be affected by the distance to the host bone.[7,8] Since new bone formation originates mainly from the existing alveolar bone at the base of the maxillary sinus, a long time is needed for the regenerated bone to reach to the Schneiderian membrane, which is distant from the osteogenic source.

There have been many attempts at sinus augmentation using recombinant human bone morphogenetic protein-2 (rhBMP-2) as an osteoinductive factor to accelerate bone regeneration,[9-12] and rhBMP-2 with an absorbable collagen sponge (ACS) was approved for clinical use in sinus augmentation and ridge augmentation by the United States Food and Drug Administration (FDA) in 2007.[13] However, because of its rapid resorption rate and lack of structural durability, the clinical use of ACS with rhBMP-2 has some limitations in sinus augmentation procedures.[14,15] A previous study evaluating the stability of NB in the rabbit sinus after filling with blood clots found that the augmented height was significantly decreased during the early

stage of bony regeneration.[16] In another previous study using a rabbit sinus model, rhBMP-2-loaded ACS also resulted in reduced augmented membrane height in spite of rapid bone regeneration rate.[15] These findings suggest that the space making ability of ACS is not sufficient for the sinus graft procedure.

Biphasic calcium phosphate (BCP) is a bone substitute material that comprises hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) in a specific ratio; its space-providing and osteoconductive properties have been well documented.[17-20] BCP has been also used as a carrier for rhBMP-2 in the alveolar ridge defect[21] and spine fusion[22,23] models. Jung *et al.*[24] evaluated rhBMP-2-loaded HA/TCP in the rabbit calvarial onlay model, and concluded that this might be an ideal carrier system for rhBMP-2.

Appropriate degradation rate of biomaterial is a requirement for the ideal carrier/scaffold. The resorption rate of BCP can be controlled by altering the ratio of HA and β-TCP. It has been found that BCP with a high ratio of HA results in successful bone regeneration in various types of osseous defect models.[20,25-27] The HA content of the scaffold can help provide space for new bone formation however, its low biodegradation rate means that the biomaterial could be left unresorbed over a prolonged period of time.[20] In contrast, a higher proportion of β-TCP in BCP increases the resorption rate, and therefore would be replaced more rapidly by NB. Ultimately, the desired

outcome is the replacement of grafted biomaterials with natural bone structures containing osteons. Therefore, in the present study we used BCP with a high β-TCP ratio as the carrier for rhBMP-2.

While soaking of BCP in a solution of rhBMP-2 is an FDA-approved loading method, there are concerns that this may result in uncontrolled flow and uneven distribution of the growth factor.[28] Kim *et al*.[28] introduced the use of BCP coated with *Escherichia-coli*-derived rhBMP-2 (ErhBMP-2), which is easy to store and handle at room temperature. In that study, particle- and block-type graft materials loaded with rhBMP-2 using the coating method exhibited enhanced bone induction in rat calvarial defects.

The purpose of the present study was to 1) elucidate the osteoinductive effect of ErhBMP-2 with BCP carrier system on grafted sinus area and surrounding tissues including the Schneiderian membrane, and 2) determine the space making ability of BCP in a standardized rabbit sinus model.

# II. Materials and methods

## 1. Animals

Thirty-six sinuses of 18 male New Zealand white rabbits weighing 2.5–3 kg were used, including 2 animals for *in vitro* test and 16 animals for *in vivo* test. Animals were housed in separate cages under standard laboratory conditions, with ad libitum access to water and a standard laboratory pellet diet. The animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea.

## 2. Preparation of ErhBMP-2-coated BCP particles

ErhBMP-2 was provided by the Cowellmedi research institute (Busan, Korea). Microporous BCP (particle type, 0.5–1.0 mm, 70% porosity; Bio-C, Cowellmedi) with a HA:β-TCP ratio of 3:7 was used as the ErhBMP-2 carrier. Details of the coating of BCP particles with ErhBMP-2 are available elsewhere.[28] Briefly, ErhBMP-2 solution (0.67 mL in 1.5 mg/mL buffer) was added to 1 g of BCP particles and lyophilized in a freeze-dryer. The solution was frozen by placing the ampoule on precooled shelves and cooling it to –

43°C. The formulations were dried in a condenser at –40°C (primary drying) and maintained at this temperature for 3 h. They were then placed in a pressure chamber at 5 mTor for 2 h. Secondary drying was performed on a shelf using the following sequence: –20°C for 4 h, –10°C for 4 h, 0°C for 2 h, and 20°C for 20 h. The chamber pressure remained constant throughout the procedure.

## 3. *in vitro* Culture

Schneiderian membranes from four normal sinuses of two animals were dissected for *in vitro* test and washed in DMEM with 10% FBS. Membranes were divided into several small pieces (3mm×3mm) and cultured *in vitro* with Trowell-type organ culture method in osteogenic medium (containing α-MEM with 15% fetal bovine serum, 2mM L-glutamine, 2mM β-Glycerophosphate, 100mM L-ascorbic acid 2-phosphate, $10^{-8}$ dexamethasone, 55mM 2-mercaptoethanol, 100U/ml penicillin and 100mg/ml streptomycin) with/without ErhBMP-2 (10 μg/ml) for two days.

## 4. Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis

RNA was extracted from Schneiderian membrane at two days after being incubated in osteogenic medium *in vitro* with or without BMP protein (with

BMP protein, *n*=8; without BMP protein: *n*=8).

RT-qPCR was performed using a Thermal Cycler Dice Real-Time System and SYBR Premix EX Taq (Takara, Japan) according to the manufacturer's instructions. PCR amplification was performed with specific primers by using standard protocols; GAPDH forward: 5'-GAGCTGAACGGGAAACTCAC-3', GAPDH reverse: 5'-CACTGTTGAAGTCGCAGGAG-3', osteocalcin (*BGLAP*-like) forward: 5'-AGAGTCTGGCAGAGGCTCA-3', osteocalcin reverse: 5'-CTCGCACACCTCCCTCTT-3', osteopontin (*SPP1*) forward: 5'-GGCTAAACCCTGACCCATCT-3', osteopontin reverse: 5'-GTGGTCATCGTCCTCATCCT-3', Type I collagen (*COL1A1*) forward: 5'-GGCGATCGTGGTGAGACT-3', Type I collagen reverse: 5'-ACCCTGGAGACCGGAGAA-3', alkaline phosphatase (*ALPL*) forward: 5'-CGTGTTCACCTTTGGAGGAT-3', alkaline phosphatase reverse: 5'-TTGTGAGCGTAGTCCACCAT-3', *RUNX2* forward: 5'-CCCTGAACTCTGCACCAAGT-3', *RUNX2* reverse: 5'-GTGCCTCGTGTGGAAGACA-3'. For RT-qPCR, the reaction mixture was initially incubated for 10 seconds at 95°C. The amplification program comprised 45 cycles of denaturation at 95°C for 5 seconds, annealing at 53-60°C for 20 seconds, and extension at 72°C for 20 seconds. The RT-qPCR for each sample was performed in triplicate and the amount of each of the RT-

qPCR products was normalized using GAPDH as an internal control. The data were analyzed with the Thermal Cycler Dice Real-Time System analysis software and the $2^{-DDCt}$ method. The statistical calculations were performed using *t*-test of variables to determine significant changes at the 95% confidence level ($P<0.05$).

## 5. *In vivo* study design and surgical procedures

The overall surgical procedure followed the protocol of our previous study.[15] General anesthesia was induced via intramuscular injection, using a mixture of ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rumpun, Bayer Korea, Seoul, Korea), and local anesthesia was administered with 2% lidocaine (lidocaine HCl, Huons, Seoul, Korea). Following shaving and local disinfection with iodine, a straight incision was made along the midline on the dorsal area of the nasal bone, and a full-thickness flap was elevated laterally to expose the nasal bone. A circular reamer (C-reamer, Neobiotech, Seoul, Korea) with a diameter of 5.5 mm that was designed to minimize membrane perforation was used to prepare the windows bilaterally. The positions of the windows were the same as those determined by Asai *et al.*[29] and Choi *et al.*[15] The prepared bony window was removed and the sinus membrane was carefully elevated. To ensure that the

same histologic position was sectioned in each animal, metal pins (Dentium, Seoul, Korea) were inserted on the nasal midline between the prepared windows, at the central-most point of each circular window (Fig. 1).

In each animal, each of the sinuses was randomly allocated to either the ErhBMP-2 or control (BCP alone) group (thus both the experimental and control groups were represented in each animal). In the ErhBMP-2 group, 0.15 g of graft material (BCP coated with ErhBMP-2) was inserted to the sinus, so that 150 μg of ErhBMP-2 was applied. In the contralateral sinus, 0.15 g of uncoated BCP was grafted as a control. After implantation of the graft material, the flap was sutured layer by layer with 4-0 monosyn (glyconate absorbable monofilament, B-Braun, Aesculap, PA, USA). The animals were sacrificed at 2 weeks ($n$ = 8) or 8 weeks ($n$ = 8) postoperatively by anesthetic overdose.

## 6. Radiographic analysis with microcomputed tomography

Block sections including the experimental site and the surrounding area were removed and immediately fixed in 10% buffered formalin for 10 days. Microcomputed tomography (micro-CT; Skyscan 1072, Skyscan, Aartselaar, Belgium) images of these block specimens were then taken at a resolution of 35 μm (100 kV and 100 μA). The scanned CT images were processed in DICOM format and three-dimensionally (3D) reconstructed with

PC-based software (On-Demand3D, Cybermed, Seoul, Korea).

Both sides of the sinuses were visualized using the threshold of 275, as mentioned in previous publications.[30] The augmented area inside of the nasal bone was identified and color-coded using the software program; the experimental and control sinuses were colored in red and blue, respectively. The volumes of the colored areas were calculated automatically (in $mm^3$).

## 7. Histologic and histomorphometric analyses

The rinsed block sections were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Serial 5-μm-thick sections were cut coronally along the center of the window. The two central-most sections were selected from each block, and stained with hematoxylin-eosin and Masson's trichrome. The histologic slides were observed and the images were digitally acquired with the aid of a light microscope (BX50, Olympus, Tokyo, Japan).

Histomorphometric measurements of the captured images were made using a PC-based image-analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The composition of the total augmented sinus was identified, and the relative areas of NB, residual materials (RM), and soft tissue (ST) were separately detected and calculated (in $mm^2$). The total area of the augmented sinus was measured and the proportions (in %) of each

composite (i.e., NB, RM, and ST) were obtained. To evaluate the distribution of regenerated bone in grafted sinus area as a secondary outcome variable, above-mentioned parameters were measured in specific standardized areas of window, central, and membrane regions. The areas were randomly selected and photomicrographs were taken in original magnification ×200. The window region was selected within the grafted area interfacing the imaginary extensions of pre-existing cortical bone at the margins of the window, and the center region was at the middle of the whole augmented sinus. The membrane region was chosen from just above the Schneiderian membrane. (Fig. 2).

## 8. Statistics

The statistical analysis was performed using a standard software program (SPSS 15.0, SPSS, Chicago, IL, USA). Independent *t*-tests were carried out to compare the results obtained from RT-qPCR in ErhBMP-2 treated and control tissues, and *in vivo* test at 2 and 8 weeks. Paired *t*-tests were used to evaluate the differences between ErhBMP-2-treated and control groups in *in vivo* tests ($p < 0.05$). The data are presented as mean±SD values.

# III. Results

## 1. Quantitative RT-PCR

Quantitative RT-PCR with RNA extracted from Schneiderian membrane of adult rabbits showed extensively increased expression of early osteoblast, such as *RUNX2*, Type I collagen, Alkaline phosphatase and Osteopontin. However, the expression level of late osteoblast like Osteocalcin was not significantly changed by BMP. (Fig. 3).

## 2. Clinical observations

During the surgical procedure, membrane perforation of less than 1 mm occurred in two sinuses from the 2-week survival group: one in the ErhBMP-2-treated group and the other in the control group. All of the rabbits recovered well and without any adverse healing events such as wound dehiscence or postoperative infection.

## 3. Radiographic analysis: micro-CT

The sinus cavity was filled with radiopaque materials in all groups.

Internally, the augmented sinus had a domed shape (Fig. 4A, B). At 2 weeks after surgery, the 3D-reconstructed appearance of the augmented sinuses differed between the experimental and control groups. The spherical shape of the grafted biomaterials was clearly evident in the control group, and the implanted materials resided within high-density. In contrast, RM were only sparsely observed in the ErhBMP-2-grafted sinus, and the surfaces of augmented sites exhibited a finely irregular appearance. At 8 weeks of healing, the appearance and surface of the augmented areas in both the experimental and control sites were similar.

The measured total augmented volume (in $mm^3$) of each group is shown in Figure 4C. At the 2-week healing point, the volume of the augmented sinus was significantly larger in the ErhBMP-2-treated group ($232.62 \pm 23.24\ mm^3$) than in the control group ($195.29 \pm 42.32\ mm^3$; $p < 0.001$). Conversely, there was no significant difference between the ErhBMP-2 and control groups at the 8-week healing point ($227.72 \pm 52.59$ vs. $223.24 \pm 17.37\ mm^3$, $p = 0.434$). The change in dimensions of the experimental sites between the two observation periods was also analyzed. The volume in the control group increased significantly as time passed ($p = 0.012$), while there was no significant difference in dimensions in the ErhBMP-2-grafted sinus, although the volume appeared to have slightly decreased at 8 weeks ($p = 0.06$).

## 4. Histologic observations

NB and RM were seen in both the ErhBMP-2 and control groups at the 2-week healing point (Fig. 5). The cross-sectional shape of the augmented sinus was convex in all animals, and the window area was not yet completely regenerated. The Schneiderian membrane was intact, with no signs of inflammation; numerous serous glands could be seen. The sinus shape in the two rabbits that experienced membrane perforation during the surgical procedure was the same as that in the unperforated rabbits.

Regenerated bone was found mostly near the sinus membrane in the ErhBMP-2 group (Fig. 5A). A trabecular pattern of NB was observed along the Schneiderian membrane and in direct contact with it. In contrast, there was no evidence of regenerated bone in the window and central regions. The RM was sparsely observed, and extensive blood vessels and connective tissues filled in the space between the biomaterials. The remaining BCP particles were irregular in shape with unclear edges (Fig. 6A, C, E).

Regenerated bone was observed all around the augmented area in the control group (Fig. 5B). However, NB was observed more extensively at the area adjacent to the existing alveolar bone rather than along the Schneiderian membrane (Fig. 6B, D, F). NB sprouted out from the lateral surface inside of the augmented sinuses. There were more BCP particles in the sinus sites of the

control group than the ErhBMP-2 group. In addition, remnant particles were larger and they were closer to each other at the control sites than at the ErhBMP-2-treated sites.

The two groups exhibited a similar pattern of bone formation and appearance of RM at the 8-weeks healing point (Fig. 7). The newly formed woven bone was replaced by lamellar bone, and a trabecular pattern was observed. The intertrabecular space was filled with RM, fibrovascular tissue and bone marrow. The window area was almost completely closed with NB, and corticalization between the prepared sites had restored the original curvature of the nasal bone.

## 5. Histomorphometric analysis

The differences in total area of augmented sinus (Fig. 8) confirmed the radiographic volumetric analysis. The total augmented area at the 2-week healing point was significantly larger in the ErhBMP-2 group (43.02 ± 7.90 $mm^2$) than in the control group (29.64 ± 6.64 $mm^2$; $p < 0.001$). At the 8-week healing point this parameter did not differ significantly between the two groups.

The results of the histomorphometric analysis in specific regions are summarized in Figure 9 and Tables 1–4. At the 2-week healing period, the

proportions of NB, RM, and ST in the totally augmented area differed between the ErhBMP-2 and control groups (Fig. 9A, Table 1). The proportions of NB and RM were greater in the control group than in the ErhBMP-2 group ($p$ = 0.013 and 0.002, respectively). Likewise, there were significant differences in the relative area of NB between the control and ErhBMP-2 groups in specific regions at the 2-week healing point (Fig. 9B–D, Tables 2–4). Typically, the results in the membrane region of all of the samples were opposite those seen in the window and central regions. Although NB was minimally observed at the window and central regions in ErhBMP-2-grafted sinuses, a significantly increased amount of NB was evident along the Schneiderian membrane of ErhBMP-2-grafted sinuses ($p$ = 0.031). At the 8-week healing point, the sinuses of the two groups were filled with similar compositions of NB, RM, and ST.

# IV. Discussion

ErhBMP-2-coated BCP particles were used in the present study to reinforce the space-making ability and enhance bone quality in sinus augmentation. Using the ACS carrier for rhBMP-2 accelerated bone regeneration, but a large amount of shrinkage was also observed in the rabbit sinus model.[15] In the present study, the grafted BCP maintained the augmented volume, with excellent NB occurring during the experimental periods. No reduction of the augmented volume was observed; rather, the total volume of sinus was increased in the control group. This finding might be associated with the increased new bone volume being added to the maintained BCP volume. Thus, BCP appears to be a better carrier than ACS with respect to volume maintenance. However, it failed to show the superiority of ErhBMP-2 coated BCP to the control in bone formation. Contrary to our expectation of accelerated bone formation in the experimental group, there was little bone formation around the ErhBMP-2-coated BCP particles at the 2-week healing point.

The differences in healing patterns between the two treatment groups at the early stage are notable. The augmented volume and area were larger in the ErhBMP-2 group than in the control group at the 2-week healing point.

These findings were attributed to postoperative swelling at the surgical site. Although many studies have shown that rhBMP-2 has osteoinductive potential, complications such as postoperative swelling have also been reported.[31-33] The amount of swelling is thought to depend upon the total dose of rhBMP-2 delivered to the surgical site.[31]

Our histologic and histomorphometric analyses revealed specific atypical features in the ErhBMP-2 group at the 2-week healing point. Little NB was found around the lateral bony wall and the grafted BCP, while most of the NB was formed along the reflected Schneiderian membrane without the intervention of BCP particles. On the other hand, newly formed trabecular bone projected from the lateral bone to the center of the sinus in the control group, and was in direct contact with the grafted BCP particles near to the parent bone. The orientation of bone healing appeared to differ between the two groups.

Some researchers have proposed that the Schneiderian membrane contains osteogenic progenitor cells, and that bone regeneration originates from the sinus membrane.[34-36] Srouji *et al*.[34] evaluated the cells derived from the human maxillary sinus membrane, and found that these cells were positive for mesenchymal stem cell markers, and underwent osteogenic differentiation both *in vitro* and *in vivo*. However, another recent *in vivo* study revealed

limited influence of Schneiderian membrane on bone formation after sinus floor elevation[37]. The results of the present study could support these two opposite studies, which showed different healing patterns around Schneiderian membrane in ErhBMP-2 treated and control sites. A seam of NB was found along the Schneiderian membrane in the experimental group of the present study, even at 2 weeks. Results from RT-qPCR also showed increased expression of early osteoblast, such as *RUNX2*, Type I collagen, Alkaline phosphatase and Osteopontin. It can be assumed that ErhBMP-2 might provoke osteogenic differentiation of progenitor cells of the Schneiderian membrane in early healing phase. From the clinical point of view, early corticalization of the outer sinus surface could protect the augmented space from the volume shrinkage by remodeling or the pressure during respiration. Therefore, it may provide a suitable environment for increasing bone formation throughout the grafted area.

On the other hand, most bone substitute materials such as BCP are osteoconductive materials that cannot induce new bone formation on their own. The trabecular bone sprouted mainly from the lateral bony wall in the control group, and less bone formation was observed around the Schneiderian membrane in the control group compared with the experimental group.

The experimental and control groups showed similar results at 8 weeks

unlike at 2 weeks where significant difference between the two groups was shown. The remaining materials were rather increased in the membrane region of ErhBMP-2 group compared to 2 weeks. It can be assumed that the new bone layer formed in the early stage in the membrane region has been resorbed due to pneumatization caused by air pressure during the remodeling process. Nevertheless, the total augmented volume was only slightly decreased because of the space making ability of BCP particles. This suggests that the ratio of remaining particles might be higher at 8 weeks in the ErhBMP-2 group.

Our results demonstrate an osteoinductive effect of ErhBMP-2 in the membrane region, but the total amount of NB was less in the ErhBMP-2 group than in the control group at the 2-week healing point, although it was similar at 8 weeks. In contrast to the membrane region, the center region of the experimental group was filled mainly with connective tissue and carrier materials. There were many blood vessels, and the density of particles was lower than that in the control group. RhBMP-2 is known to induce both angiogenesis and osteogenesis, through chemotaxis of osteoblasts and endothelial cells.[38] It is inferred that swelling by angiogenesis and chemotaxis of rhBMP-2 may have resulted in the relatively sparse presence of the particles.

It is unclear why new bone formation did not occur around the graft particles in the experimental group at 2 weeks. One possibility is that the high

dose of rhBMP-2 impaired healing. The effective dose of BMP-2 is known to differ between species and in various types of animal model. In general, a much smaller amount of rhBMP-2 is sufficient to induce bone formation in small animals such as rabbits than in human beings. BCP particles coated with 1.5 mg/mL ErhBMP-2 solution were grafted in this study; it is possible that this constitutes an overdose in rabbits. Zara *et al.*[33] reported on the adverse effects of high-dose BMP-2. They found that BMP-2 had dose-dependent side effects, such as cyst-like bone void formation. In the present study it can be assumed that the membrane region spatially distant from the particles was stimulated by the diluted signal of the released rhBMP-2, while osteogenic differentiation was inhibited at the area near to the particle due to the high concentration of rhBMP-2. The other possibility is that the method used to load the rhBMP-2 molecule onto the BCP particles in the present study—whereby rhBMP-2 solution was lyophilized to the BCP particle surface—or the process of its release altered the biological activity of the rhBMP-2 molecules. Further study is required to clarify these potentially adverse effects.

# V. Conclusion

Within the limitations of the study, it can be concluded that BCP exhibits good space maintenance in sinus augmentation, and that ErhBMP-2 may stimulate the osteoinductive potential of the sinus membrane at the early stage of healing, although there was no significant superiority in ultimate bone formation.

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# Legends

**Figure 1.** Surgical procedure. **(A)** Windows were prepared bilaterally, and graft materials were inserted. A metal pin was applied to the midline suture to enable identification of the middle of the windows. **(B)** A circular reamer with a diameter of 5.5 mm was used to minimize sinus membrane perforation.

**Figure 2.** Histomorphometric analysis. The proportions of NB and graft RM were measured on the total augmented area (dotted line), and three randomly selected regions (a, window; b, center; c, membrane).

**Figure 3.** Osteoblastic gene transcriptional changes in Schneiderian membrane after treatment of ErhBMP-2. Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis shows the significant upregulation of *RUNX2*, Type I collagen (*COL1A1*), Alkaline phosphatase (*ALPL*), *Osteopontin* (*SPP1*) after treatment of ErhBMP-2 for 48 hours (asterisks, $p<0.05$). The expression level of *Osteocalcin* (*BGLAP*-like) was not evidently changed after ErhBMP-2 treatment. Error bars indicate standard deviations on the normalized ratio.

**Figure 4.** Radiographic analysis. The augmented sinuses were identified and color-coded on 3D-reconstructed micro-CT images. The experimental (ErhBMP-2-treated) sinus was coded in red, and the contralateral, control (BCP-alone) sinus was coded in blue. **(A)** After 2 weeks of healing. **(B)** After 8 weeks of healing. (C) Total augmented volume. At the 2-week healing point, the total augmented volume was much larger in the ErhBMP-2 group than in the control group ($p < 0.001$). In the control group, the volume was increased at the 8-week healing point ($p = 0.012$).

**Figure 5.** Histologic findings after 2 weeks of healing. NB and RM were seen in both groups. The surgically created windows were clearly distinguished (arrowheads). **(A)** ErhBMP-2 group. The regenerated bone was found mostly along the sinus membrane. In contrast, there was no bone regeneration in the window and center areas. **(B)** Control group. The NB originating from the native bone was seen throughout the augmented area (Masson trichrome stain).

**Figure 6.** Histologic findings after 2 weeks of healing. The regenerated sinus was divided into three regions: window **(A, B)**, center **(C, D)**, and membrane **(E, F)**. **(A, C, E)** ErhBMP-2 group. No bone was found in the window and center regions. On the other hand, respectable bone formation was observed

along the Schneiderian membrane without the intervention of BCP materials. **(B, D, F**) Control group. NB and BCP particles were seen in all regions. A sprouting out of NB was seen, and NB occurred in direct contact with the BCP particles (arrowhead: window site; A–D, Masson's trichrome; E, F, hematoxylin-eosin).

**Figure 7.** Histologic findings after 8 weeks of healing. The NB was lamellar bone, and a trabecular pattern was observed. The intertrabecular space was filled with RM, fibrous tissue, and bone marrow. The window area was almost completely closed. **(A)** ErhBMP-2 group. **(B)** Control group (Masson's trichrome).

**Figure 8.** Total area. The total augmented area was larger in the ErhBMP-2 group than in the control group at the 2-week healing point ($p < 0.001$), but did not differ between the two groups at the 8-week point.

**Figure 9.** Fraction of augmented area. **(A)** Total area. **(B)** Window region. **(C)** Center region. **(D)** Membrane region.

# Tables

TABLE 1. COMPOSITION OF THE TOTAL AUGMENTED AREA (%, MEAN±SD)

| *Healing period* | | *NB* | *RM* | *ST* |
|---|---|---|---|---|
| 2 weeks | | | | |
| ErhBMP-2 | Area ($mm^2$) | 0.74 ± 0.50 | 6.22 ± 3.30 | 36.07 ± 5.17 |
| | Ratio (%) | 1.63 ± 0.88[a,b] | 13.96 ± 4.96[b] | 84.41 ± 5.15[b] |
| Control | Area ($mm^2$) | 1.02 ± 0.31 | 6.55 ± 3.29 | 22.07 ± 4.90 |
| | Ratio (%) | 3.57 ± 1.19 | 21.53 ± 7.73 | 74.91 ± 7.25 |
| 8 weeks | | | | |
| ErhBMP-2 | Area ($mm^2$) | 2.92 ± 1.01 | 5.86 ± 2.77 | 30.79 ± 5.05 |
| | Ratio (%) | 9.39 ± 2.21 | 14.38 ± 5.07 | 76.23 ± 5.77 |
| Control | Area ($mm^2$) | 3.93 ± 1.90 | 4.73 ± 1.35 | 27.92 ± 7.79 |
| | Ratio (%) | 10.82 ± 4.51 | 13.35 ± 3.74 | 75.83 ± 4.49 |

[a]Significantly different from the group with the same protocol at 8 weeks ($p < 0.05$).

[b]Significantly different from the group with control group at the same observation period ($p < 0.05$)

NB, newly formed bone; RM, residual materials; ST, soft tissue; ErhBMP-2, *Escherichia-coli*-derived rhBMP-2.

TABLE 2. COMPOSITION OF THE WINDOW REGION (%, MEAN±SD)

| *Healing period* | *NB* | *RM* | *ST* |
|---|---|---|---|
| 2 weeks | | | |
| ErhBMP-2 | 0.06 ± 0.18[a,b] | 46.18 ± 15.38 | 53.75 ± 15.75[b] |
| Control | 7.27 ± 5.09 | 56.78 ± 6.74 | 35.95 ± 3.89 |
| 8 weeks | | | |
| ErhBMP-2 | 19.97 ± 5.58 | 52.74 ± 9.98 | 27.29 ± 7.27 |
| Control | 26.81 ± 10.42 | 43.81 ± 7.10 | 29.37 ± 5.71 |

[a]Significantly different from the group with the same protocol at 8 weeks ($p < 0.05$).

[b]Significantly different from the group with control group at the same observation period ($p < 0.05$).

TABLE 3. COMPOSITION OF THE CENTER REGION (%, MEAN±SD)

| *Healing period* | *NB* | *RM* | *ST* |
|---|---|---|---|
| 2 weeks | | | |
| ErhBMP-2 | 0[a,b] | 56.82 ± 7.27 | 43.18 ± 7.27[a] |
| Control | 7.41 ± 5.21 | 54.35 ± 6.39 | 38.24 ± 7.55 |
| 8 weeks | | | |
| ErhBMP-2 | 17.69 ± 6.83 | 50.63 ± 7.84 | 31.68 ± 2.39 |
| Control | 22.14 ± 7.83 | 45.73 ± 7.73 | 32.12 ± 9.49 |

[a]Significantly different from the group with the same protocol at 8 weeks ($p < 0.05$).

[b]Significantly different from the group with control group at the same observation period ($p < 0.05$).

TABLE 4. COMPOSITION OF MEMBRANE REGION (%, MEAN±SD).

| *Healing period* | *NB* | *RM* | *ST* |
|---|---|---|---|
| 2 weeks | | | |
| ErhBMP-2 | 20.86 ± 12.05[a] | 13.38 ± 11.03[a,b] | 65.76 ± 8.40[a] |
| Control | 15.58 ± 4.33 | 48.76 ± 7.46 | 35.65 ± 6.06 |
| 8 weeks | | | |
| ErhBMP-2 | 29.33 ± 12.05 | 43.67 ± 5.76 | 27.00 ± 5.50 |
| Control | 28.17 ± 7.94 | 41.42 ± 12.63 | 30.40 ± 7.01 |

[a]Significantly different from the group with control group at the same observation period ($p < 0.05$).

[b]Significantly different from the group with the same protocol at 8 weeks ($p < 0.05$).

# Figures

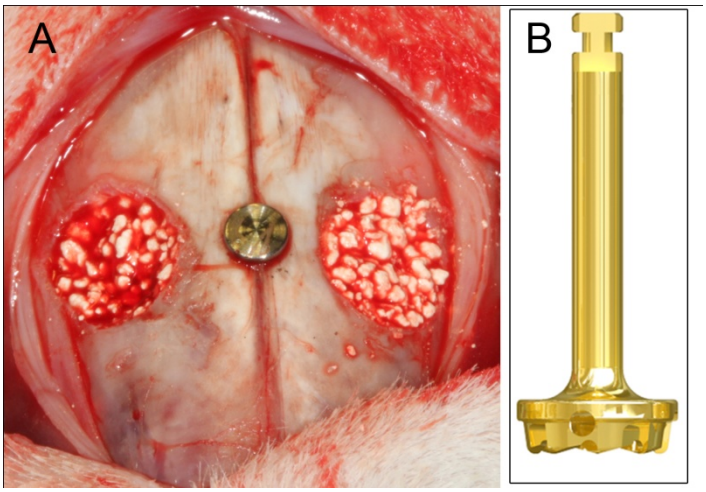



**Figure 1**

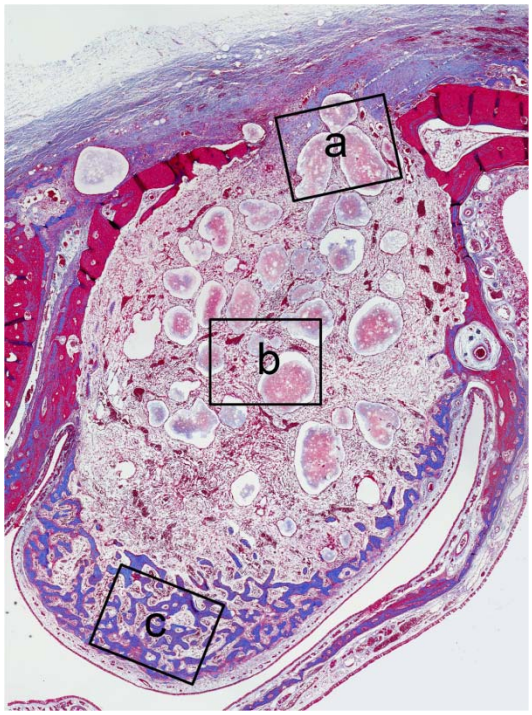



**Figure 2**

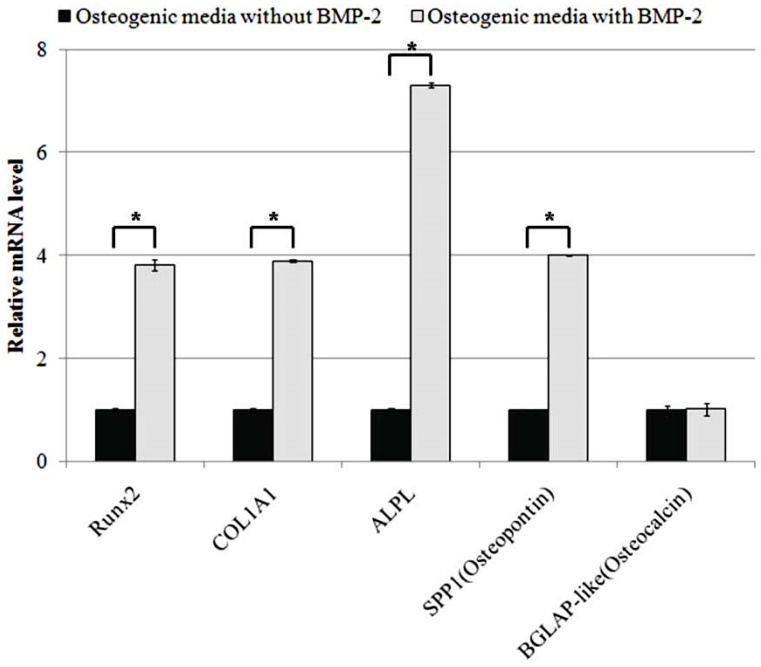

Osteogenic media without BMP-2
Osteogenic media with BMP-2
Relative mRNA level
8
6
4
2
0
*
*
*
*
Runx2
COL1A1
ALPL
SPP1(Osteopontin)
BGLAP-like(Osteocalcin)

**Figure 3**

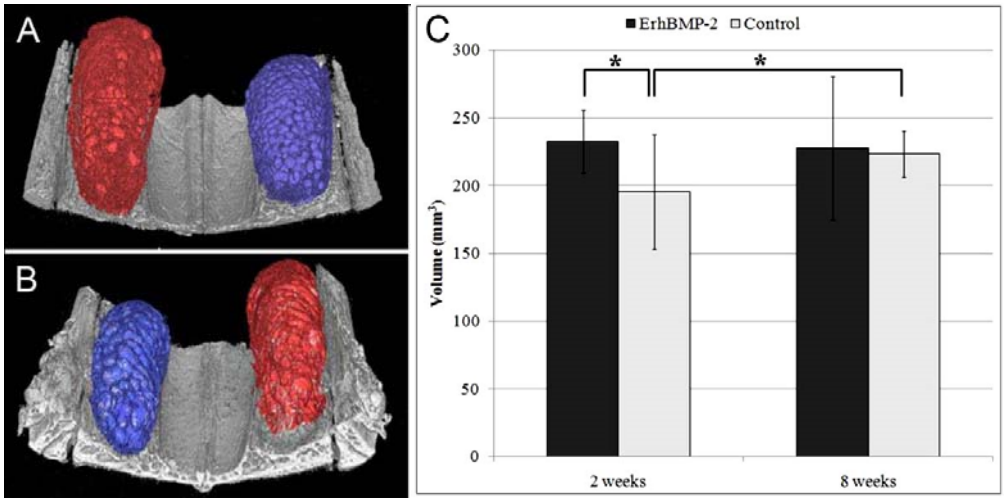

A
B
C
ErhBMP-2
Control
300
250
200
150
100
50
0
Volume (mm³)
*
*
2 weeks
8 weeks

**Figure 4**

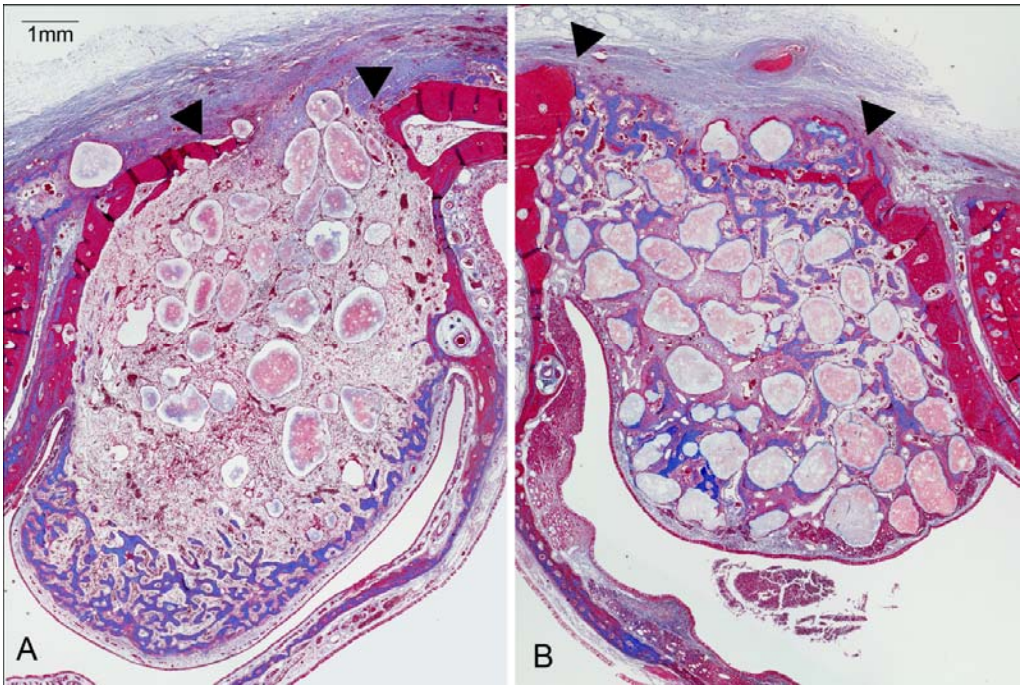
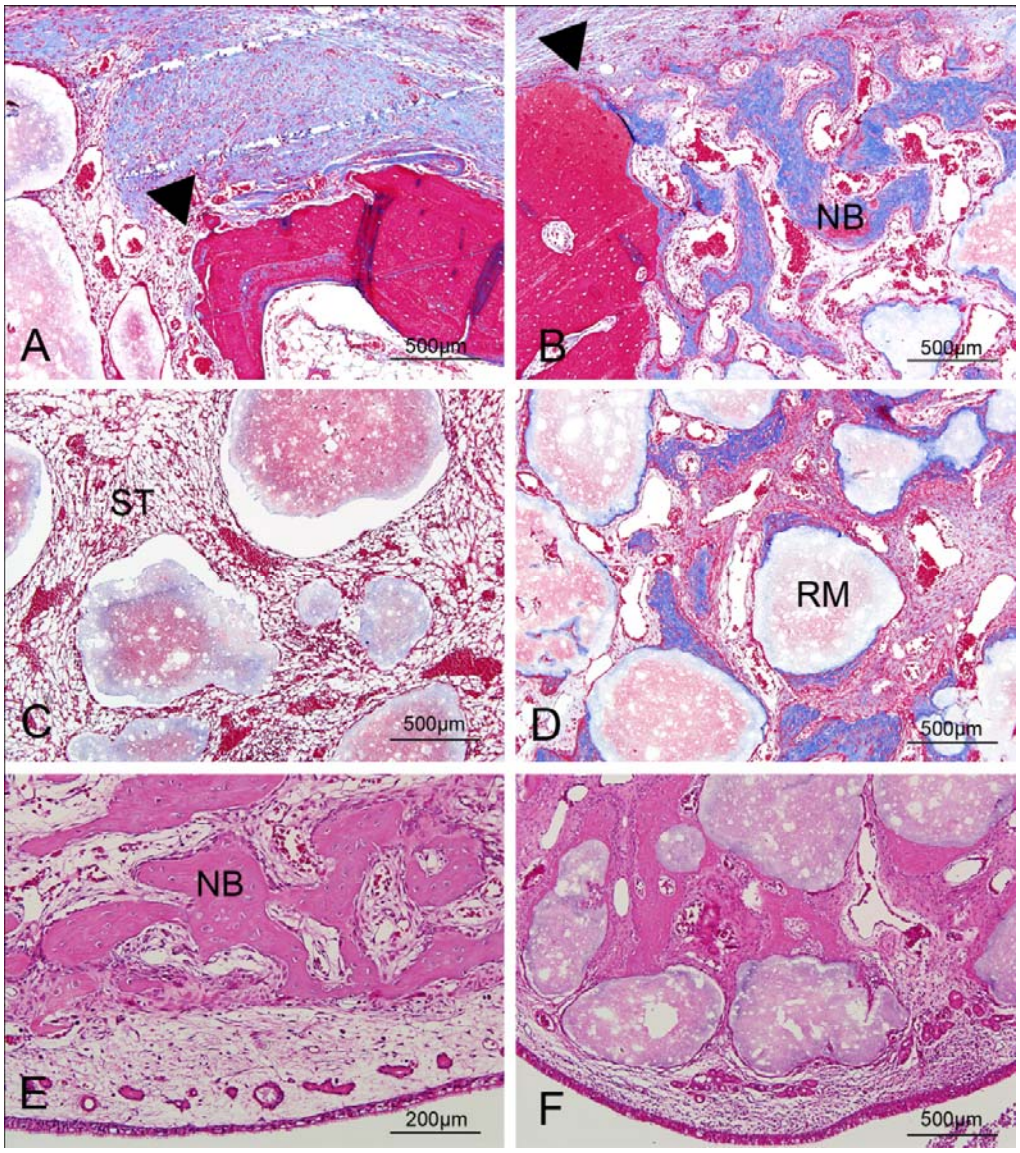



**Figure 5**

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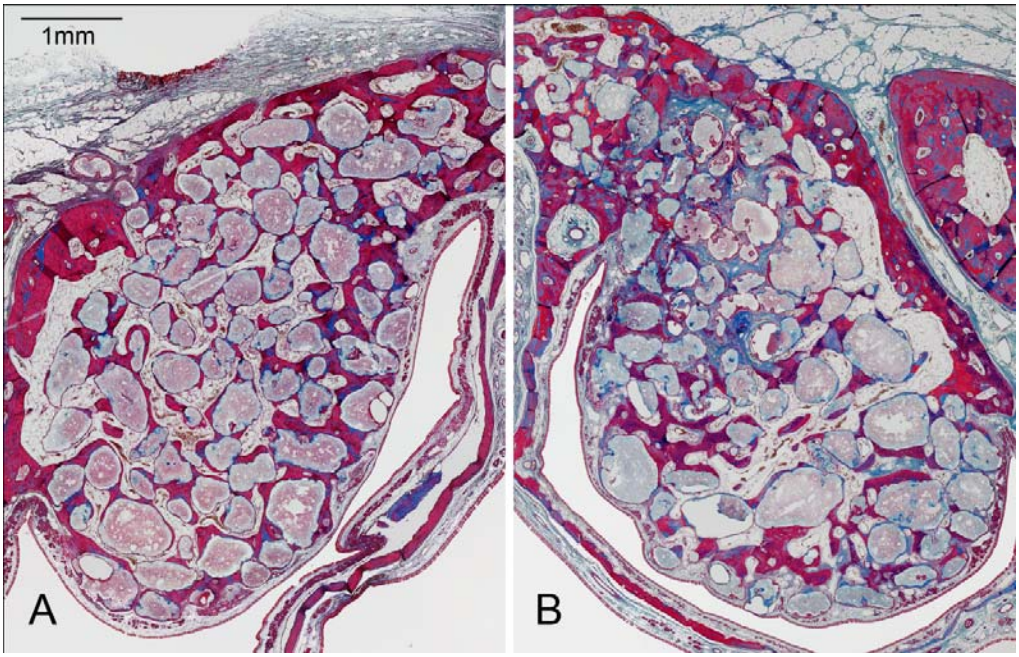


**Figure 7**

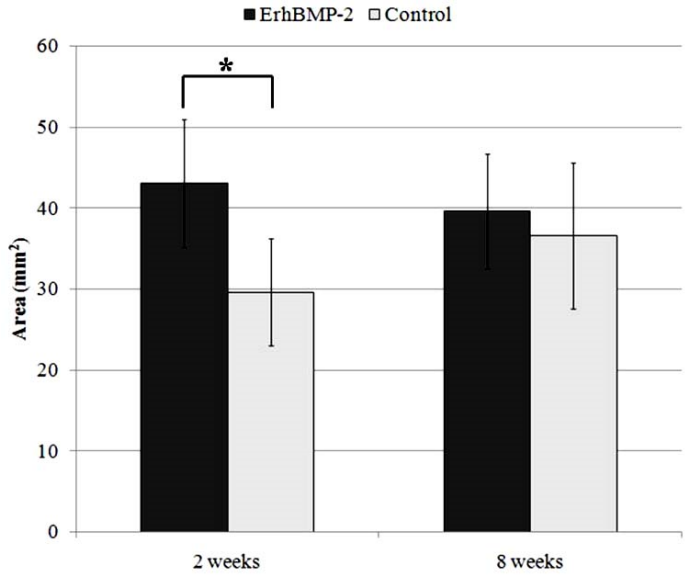


**Figure 8**

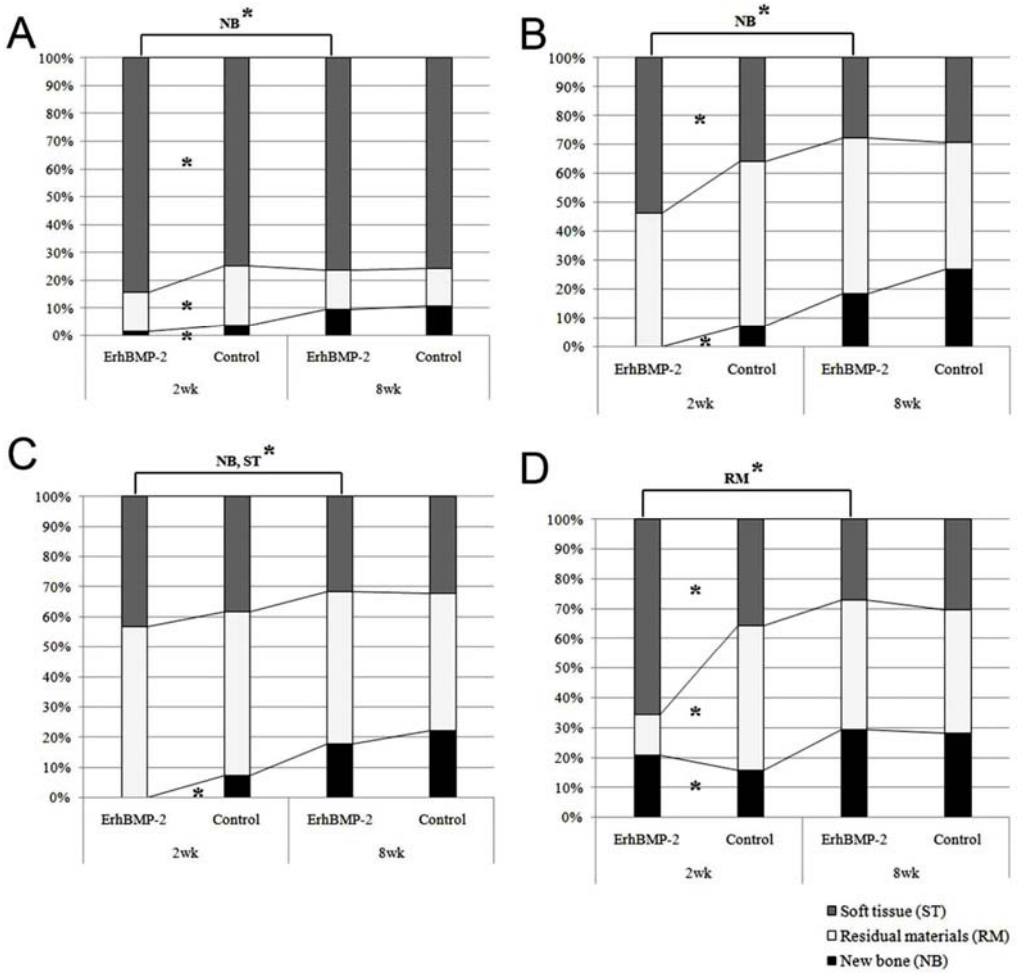
A
NB *
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
ErhBMP-2
Control
ErhBMP-2
Control
2wk
8wk
B
NB *
C
NB, ST *
D
RM *
Soft tissue (ST)
Residual materials (RM)
New bone (NB)

**Figure 9**

국문요약

# 제 2형 재조합 인간 골형성 단백질이 상악동 막의 골형성 능력에 미치는 영향 : 토끼 상악동에서의 조직계측학적 연구

<지도교수 **최 성 호**>

연세대학교 대학원 치의학과

**최 연 아**

이 연구는 재조합 인간 골형성 단백질-2 (recombinant human bone morphogenetic protein-2; rhBMP-2) 와 이상인산칼슘 (biphasic calcium phosphate; BCP) 전달체를 상악동에 이식했을 때 상악동막을 포함한 주위 조직에 미치는 영향을 평가하는 것이다.

총 18 마리의 토끼를 이용하여 실험을 하였으며, 이 중 2 마리는 실험실 연구, 16 마리는 생체 연구를 위하여 이용하였다. 두 마리의 토끼에서 채취한 상악동막은 rhBMP-2 를 적용하거나, 적용하지 않은 환경에서 배양한 후 정량적 역전사 중합효소 연쇄 반응을 시행해 분석하였다. 16 마리의 토끼는 rhBMP-2 와 BCP 의 상악동의 증강 및 골재생능력을 평가하기 위해 상악동 골이식술을 시행하였다. 각각의 토끼의 양측 상악동에 이식재를 적용하였으며, 무작위로 배정된 한 쪽 상악동에는 rhBCP-2 가 코팅된 BCP 를 이식하고 (실험군), 다른 한 쪽 상악동에는 BCP 만을 이식하였다 (대조군). 수술 2 주, 8 주 후 각 8 마리씩 희생하여 방사선학적, 조직학적인 계측을 시행하였다.

채취된 상악동막을 배양한지 2 일 후, rhBMP-2 를 적용한 군에서 초기 골모세포의 발현 (RUNX2, Type I collagen, Alkaline phosphataase,

Osteopontin)이 유의성 있게 증가한 양상이 관찰되었다. 상악동 골이식술 2 주 후 실험군과 대조군의 조직학적 양상에서 차이를 보였다: 실험군에서는 상악동 막을 따라 상당한 양의 신생골이 재생되었으나 창 부위와 중앙부에는 신생골을 거의 관찰할 수 없는 반면 (window = 0.06%; center = 0%; membrane = 20.86% of new bone), 대조군에서는 모든 부위에서 균일하게 신생골이 재생된 양상을 보였다 (window = 7.27%; center = 7.41%; membrane = 15.58% of new bone). 증강된 상악동의 부피는 수술 2 주, 8 주 후에 모두 양호하게 유지되는 결과를 보였으나, 2 주군에서는 대조군에 비해 실험군의 부피가 유의성 있게 증가한 것이 관찰되었다 (232.62 $mm^3$ and 195.29 $mm^3$, respectively; $p < 0.001$).

이상의 연구를 통해, BCP 이식체는 이식 후 증강된 상악동의 부피를 양호하게 유지하며, rhBMP-2 가 적용될 경우 초기 골형성 과정에서 상악동막의 골형성능을 활성화시키는 것으로 결론 지을 수 있다.

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**핵심되는 말** : 재조합 인간 골형성 단백질-2, 이상 인산 칼슘, 상악동막, 골재생