저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer
Rapid bone regeneration
by *Escherichia coli*-derived recombinant human
bone morphogenetic protein-2 loaded
on a hydroxyapatite carrier
in the rabbit calvarial defect model

Chung-Hoon Chung

The Graduate School
Yonsei University
Department of Dental Science
Rapid bone regeneration by *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 loaded on a hydroxyapatite carrier in the rabbit calvarial defect model

Directed by Professor Seong-Ho Choi

The Doctoral Dissertation submitted to the Department of Dentistry the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Ph.D. in Dental Science

Chung-Hoon Chung

June 2016
This certifies that the dissertation thesis of Chung-Hoon Chung is approved.

Thesis Supervisor: Seong-Ho Choi

Ui-Won Jung

Jung-Seok Lee

Eun-Kyoung Pang

Young-Taek Kim

The Graduate School

Yonsei University

June 2016
감사의 글

본 논문이 완성되기까지 부족한 저를 항상 격려해 주시고 사랑과 관심으로 논문지도를 해주신 최성호 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 따뜻한 관심으로 지켜봐 주신 방은경 교수님께도 진심으로 감사 드립니다. 논문 성사를 맡아주신 정의원 교수님, 이용식 교수님, 김영백 교수님께도 감사드립니다.

실험 및 연구를 도와준 김유경 선생님, 윤소라 선생님을 비롯한 의국원 후배님들에게도 감사드립니다. 무엇보다도 현실적인 내조와 아낌없는 사랑으로 많은 힘이 되어준 아내와 결에 있는 것만으로 행복을 주는 딸 서현이에게도 진정으로 사랑과 고마움의 마음을 전합니다. 항상 격려해 주신 양가부모님, 큰누나, 작은누나, 양가 친척어르신들 그밖에도 일일이 열거하지 못하지만 응원해주셨던 모든분들에게 감사드리며 앞으로 일신우일신하는 좋은 모습으로 보답하겠습니다.

2016년 7월
저자 쓸
# Table of Contents

Abstract (English) ................................................................. iii
I. Introduction ............................................................................ 1

II. Materials and Methods .......................................................... 5
   1. Animals ........................................................................... 5
   2. Materials ......................................................................... 6
   3. Study design ..................................................................... 6
   4. Surgical protocol ............................................................. 6
   5. Histological processing ...................................................... 7
   6. Clinical observation ........................................................... 7
   7. Histological observation ..................................................... 8
   8. Histomorphometric observation .......................................... 8
   9. Tomographic analysis ....................................................... 9
  10. Statistical analysis ............................................................. 9

III. Results .................................................................................. 10
   1. Clinical findings .............................................................. 10
   2. Histologic findings .......................................................... 10
   3. Histomorphometric findings .............................................. 11
   4. Tomographic analysis ....................................................... 11

IV. Discussion .......................................................................... 15

V. Conclusion .......................................................................... 21

References .............................................................................. 22

Figure Legends ......................................................................... 29

Figures ..................................................................................... 31

Abstract(Korean) ................................................................. 38
List of Figures

Figure 1. Two circular intraosseous defects of 8 mm diameter were made in each rabbit calvarium ........................................ 29
Figure 2. Representative photomicrographs obtained at 2 weeks postoperation ⋅⋅⋅ 29
Figure 3. Representative photomicrographs obtained at 8 weeks postoperation ⋅⋅⋅ 30
Figure 4. Representative coronally sectioned micro-computed tomography images at 2 weeks postoperation .............................. 30
Figure 5. Representative coronally sectioned micro-computed tomography images at 8 weeks postoperation ................................... 31
Figure 6. Graphs showing histomorphometric analysis of total augmented area and new bone area (mm$^2$) ......................... 31
Figure 7 Graphs showing histomorphometric analysis of total augmented area and new bone volume (mm$^3$) .................. 32

List of Tables

Table 1. Total augmented area of each group (histomorphometric analysis) ⋅ 14
Table 2. New bone area of each group (histomorphometric analysis) ⋅⋅⋅ 14
Table 3. Residual particle area of each group (histomorphometric analysis) ⋅⋅⋅ 14
Table 4. Total augmented volume of each group (tomographic analysis) ⋅⋅⋅ 15
Table 5. New bone volume of each group (tomographic analysis) ⋅⋅⋅⋅⋅ 15
Abstract

Rapid bone regeneration by *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 loaded on a hydroxyapatite carrier in the rabbit calvarial defect model

The aim of this study was to determine the osteoconductivity of hydroxyapatite particles (HAP) as a carrier for *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (ErhBMP-2). Two 8-mm diameter bicortical calvarial defects were created in each of 20 rabbits. One of each pair of defects was randomly assigned to be filled with HAP only (HAP group) or ErhBMP-2 loaded HAP (ErhBMP-2/HAP group), while the other defect was left untreated (control group). The animals were killed after either 2 weeks (n = 10) or 8 weeks (n = 10) of healing, and histological, histomorphometric, and tomographic analyses were performed.

All experimental sites showed uneventful healing during the postoperative healing period. In both histomorphometric and tomographic analyses, the new bone area or volume of the ErhBMP-2/HAP group was significantly greater than that of the HAP and control groups at 2 weeks (p < 0.05). However, at 8 weeks, no significant difference in new bone area or volume was observed between the ErhBMP-2/HAP and HAP groups. The total augmented area or volume was not significantly different between the ErhBMP-2/HAP and HAP groups at 2 and 8 weeks.

Combining ErhBMP-2 with HAP could significantly promote rapid initial new bone formation. Moreover, HAP graft could increase new bone formation and space maintenance, therefore it might be one of the effective carriers of ErhBMP-2.

**Key Words:** *Escherichia coli*-derived recombinant human bone morphogenetic protein-2; hydroxyapatite; bone regeneration; tissue engineering; calvarial intraosseous defect model
Rapid bone regeneration by *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 loaded on a hydroxyapatite carrier in the rabbit calvarial defect model

Chung-Hoon Chung, D.D.S., M.S.D.

*Department of Dental Science*

*Graduate School, Yonsei University*

*(Directed by Prof. Seong-Ho Choi, D.D.S., M.S.D., PhD.)*

I. Introduction

Bone augmentation is generally carried out using autogenous bone, allograft, xenograft, or alloplastic materials. The ideal bone graft includes elements that are osteogenic, osteoinductive, and osteoconductive. Autogenous bone contains all three types of elements, but it is not available in every situation. Many studies have shown that xenograft or alloplastic materials augmented with growth factors improve bone regeneration, a major focus of tissue engineering. Numerous growth factors that
enhance various types of cell migration, adherence, and proliferation have been identified. One of these, bone morphogenetic protein (BMP), is a multifunctional protein with a wide range of biological activities in a variety of cell types (Ebra et al., 2002). BMPs regulate growth, differentiation, chemotaxis, and apoptosis. They also play pivotal roles in morphogenesis (Hogan et al., 1996). BMPs constitute the osteoinductive component of several tissue engineering products that are used in late-stage development as replacements for autogenous bone grafts and for bone augmentation and repair (Wozney et al., 2002). Many studies support the use of recombinant human BMP-2 (rhBMP-2) (Herford et al., 2008; Lan et al., 2007). However, BMP-2 derived from Chinese hamster ovary cells (CHO BMP-2) is relatively costly because protein yields are low. In this context, our research group recently succeeded in producing BMP-2 using an Escherichia coli production system (ErhBMP-2), which is particularly attractive for biotechnology because of the ability of E. coli to grow rapidly and to high density on inexpensive substrates (Ono et al., 2014). Moreover, ErhBMP-2 and CHO BMP-2 may function similarly in bone regeneration (Bessho et al., 2000). Several studies have demonstrated the efficacy of ErhBMP-2; for example, it was shown that ErhBMP-2 facilitated closure of the bone gap of a sinus window (Choi et al., 2012) and ErhBMP-2-coated implants enhanced bone-to-implant contact (Lee et al., 2013). Various carriers have been recommended, including fibrin-fibronectin, biphasic calcium phosphate, beta-tricalcium phosphate.
(β-TCP), and hydroxyapatite (HA) (Hong et al., 2006). The biological response to bone substitute materials depends not only on their chemical composition but also on their macro- and microstructural characteristics, including pore size, porosity, and interconnectivity (Hannink et al., 2011). The United States Food and Drug Administration has approved the use of BMP with an absorbable collagen sponge as carrier. However, ErhBMP-2 can be separated from the collagen under physical pressure, and collagen is rapidly absorbed. The carrier needs to be able to maintain space for subsequent bone formation. One carrier with this ability is HA. HA is used as a bone graft extender for posterolateral spinal fusion in humans (Annis et al., 2015). It is also useful as an ErhBMP-2 carrier because of its high affinity for ErhBMP-2. ErhBMP-2-adsorbed hydroxyapatite particles (HAP) are safe and can be an effective and attractive material for bone formation, since the pore size of HAP is approximately 100–300 μm. The optimal pore size for bone regeneration is known to be 300–400 μm. The minimal interconnection pore size is 5–15 μm for fibrous tissue, 40–100 μm for osteoid tissue, and 100 μm for mineralized bone. Therefore, it appears that the pore size of HAP is suitable for promoting early bone ingrowth. Furthermore, in another study, alkaline phosphatase activity was significantly higher in mandibular defects treated with porous HA and ErhBMP-2 than in controls treated with HA alone at both 7 and 21 days (Yoshida et al., 1999), indicating that ErhBMP-2 accelerated bone formation by osteoconduction from porous HA.
β-TCP is more bioresorbable than HAP and is replaced by new bone at a high rate (Jensen et al., 2006). In a study that compared changes in the distribution and expression of biomarkers of reactogenicity in the lower jaws of rabbits after implantation, osteoblast proliferation and regions of granulation tissue formation were more noticeable in experimental tissues than in the control tissue. The experimental and control groups did not differ significantly in mean β-defensin-2, IL-1, IL-6, IL-8, IL-10, osteopontin, osteocalcin, BMP-2/4, or osteoprotegerin expression. Furthermore, the prevalence of osteopontin- and osteocalcin-positive osteocytes in experimental tissues implanted with HAP at 3 months after implantation indicated potential bone regeneration stimulated by pure HAP. Therefore, the slow resorption of HAP may enhance osteoconductivity, thereby promoting new bone growth. Based on these studies, we aimed to evaluate the effect of HAP on bone regeneration and to determine the efficacy of HAP as a carrier for ErhBMP-2 in the rabbit calvarial intraosseous defect model.
II. Materials & methods

1. Animals

Twenty male New Zealand white rabbits (age, 9–20 months; body weight, 3–3.5 kg) were used in this study. The animals were housed in divided cages under standard laboratory conditions and fed a standard diet. The selection of experimental animals, their management, and the surgical protocol followed routines approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea.

2. Materials

Large amounts of BMP-2 are difficult to purify or produce in vitro using eukaryotic cells. Human recombinant BMP-2 produced in E. coli is a homodimeric, non-glycosylated polypeptide containing $2 \times 115$ amino acids, with a molecular mass of 26 kDa. The ErhBMP-2 used in this study was provided by Daewoong Pharmaceutical Co., Ltd. (Novosis®-dent, Gyeonggi, South Korea). Lyophilized BMP-2 was dissolved in 10 cm$^3$ of distilled water to yield a concentration of 0.1 mg/mL. HAP, manufactured by BioAlpha Inc. (Bongros®, Gyeonggi, South Korea), was used as the carrier material. Bongros® is composed of pure HAP and has a
particle diameter of 0.6–1.0 mm. Bongros® was loaded with ErhBMP-2 by soaking
0.1 g of the material in 0.15 mL of ErhBMP-2 solution for 10 minutes. An ErhBMP-2
dose of 1.5 μg was achieved.

3. Study design

Two circular calvarial intraosseous defects (8 mm in external diameter) were
created side by side. Rabbits were divided into two treatment groups: (1) HAP only
and (2) ErhBMP-2-loaded HAP (n = 10 animals per group). In each animal, graft
materials were grafted into one of the defects, while the other defect was designated a
sham surgery control and was filled with blood clots alone. The experimental sites for
introduction of HAP or ErhBMP-2-loaded HAP were randomly allocated. The
surgeon was not informed of the allocation until the defects had been created.

4. Surgical protocol

Rabbits were anesthetized with an intramuscular injection of a 4:1 solution of
ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rompun, Bayer
Korea, Seoul, Korea). The surgical site was shaved and disinfected with povidone
iodine, and then infiltration anesthesia was induced by injection of 2% lidocaine
(lidocaine-HCl, Huons, Seoul, Korea). An incision was made in the sagittal plane, and a full-thickness flap was elevated. The two circular defects were then created in each animal using 8-mm trephines under cool saline irrigation. The distance between the defects was 3 mm (Figure 1). The assigned graft material was grafted into one of the defects. The soft tissue was repositioned and then sutured layer-by-layer using 4-0 synthetic absorbable multifilament suture materials (VicrylPlus Antibacterial, Ethicon, Somerville, NJ, USA). Postoperative antibiotics (gentamicin; 5 mg/kg body weight) were administered by daily intramuscular injection for 1 week. The rabbits were killed at either 2 weeks (n = 5 per group) or 8 weeks (n = 5 per group) post-surgery.

5. Histological processing

Blocks that included the adjacent tissues were harvested. The blocks were fixed in 10% buffered formalin for 10 days, decalcified in 5% formic acid for 14 days, and then embedded in paraffin. Serial sections of 5-μm thickness were cut. The two center-most sections were selected from each block and stained with hematoxylin and eosin.

6. Clinical observation
Animals were carefully observed for inflammation, allergic reactions, and other complications surrounding the surgical site throughout the 2- and 8-week postoperative healing periods.

7. Histological observation

Specimens were examined under a microscope (DM LB, Leica Microsystems, Wetzlar, Germany) equipped with a camera (DC300F, Leica Microsystems) by a single, blinded examiner. Images of the slides were acquired and saved as digital files. Sections were examined at a magnification of 40×.

8. Histomorphometric analysis

Histomorphometric data for the following parameters were obtained with an automated image-analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA; Figure 2, 3):

(1) Total augmented area (mm$^2$): the area of all tissues between the defect margins, including new bone, connective tissue, and vessels; (2) New bone area (mm$^2$): the area of newly formed bone within the total augmented area; and (3) Residual particle area (mm$^2$): the area of HAP remaining within the defect.
9. Tomographic analysis

Specimens were scanned using a microcomputed tomography (micro-CT) system (SkyScans1072, SkyScan, Aartselaar, Belgium) at a resolution of 18 µm (100 kV and 100 µA) (Figure 4, 5). The scanned sets of data were processed in DICOM format, and the sum of the cross-sectional view was used to reconstruct the area of interest (Guda et al., 2014). The overall dimensional topography of the recipient beds was measured in the reconstructed views.

10. Statistical analysis

Statistical analysis was performed using a commercially available software program (SPSS 15.0, SPSS, Chicago, IL, USA). Data from the histological and three-dimensional micro-CT sections are presented as mean ± standard deviation. The Kruskal–Wallis test was used to compare the control, HAP only, and ErhBMP-2-loaded HAP groups. The Mann–Whitney U test was used to compare samples collected at 2 weeks and 8 weeks post-surgery. The level for statistical significance was set at \( p < 0.05 \).
III. Results

1. Clinical findings

All experimental sites showed uneventful healing during the postoperative healing period. No evidence of complications, such as abnormal bleeding, infection, or exposure of graft materials, was observed. Signs of inflammation, such as swelling, were minimal, and the grafted materials were confirmed to be intact within the defects at the time of sacrifice and sample collection.

2. Histologic findings

After 2 weeks of healing, the sham surgery control defects in the HAP only and ErhBMP-2/HAP groups showed a small amount of wedge-shaped new bone formation, limited to the defect margin. The amount of newly formed bone in defects with grafted material was greater in the ErhBMP-2/HAP group than in the HAP only group. The center of control defects was depressed, and thus flattened, by surrounding connective tissue and dura mater. In contrast, the center of HAP only and ErhBMP-2/HAP defects was elevated by the grafted material. New bone was formed at the
defect margins (Figure 2). After 8 weeks of healing, the HAP only and ErhBMP-2/HAP groups showed similar amounts of newly formed bone (Figure 3). More newly formed bone was generated in the center of the defects during the 8-week healing period than during the 2-week healing period. The residual particle area was not reduced after 8 weeks of healing compared with 2 weeks of healing (Figure 2, 3).

3. Histomorphometric findings

The histomorphometric measurements are summarized in Tables 1, 2, and 3. The total augmented area was significantly greater in the HAP only and ErhBMP-2/HAP defects than in controls at 2 and 8 weeks (Figure 6). Within each group, no significant difference in total augmented area was observed between 2 and 8 weeks (Table 1). At 2 weeks, the area of new bone differed significantly between the HAP only and ErhBMP-2/HAP defects (Table 2). The amount of residual material was similar between HAP only and ErhBMP-2/HAP defects at 2 and 8 weeks (Table 3).

4. Tomographic analysis

The overall dimensional topography of the defects and grafts was measured in reconstructed views at 2 and 8 weeks (Figure 4, 5). Newly formed bone was gray,
while HAP was white because of radiopacity. The total augmented volume and new 
bone volume of each group were measured using micro-CT (Table 4, 5). At 2 and 8 
weeks, the total augmented volume was significantly greater in the HAP only and  
ErhBMP-2/HAP groups than in controls (Figure 7). However, the total augmented 
volume did not differ significantly between the HAP only and ErhBMP-2/HAP 
groups at either 2 or 8 weeks (Table 4). At 2 and 8 weeks, the new bone volume was 
significantly greater in the HAP only and ErhBMP-2/HAP groups than in controls 
(Figure 7). At 2 weeks, the new bone volume was significantly greater in the  
ErhBMP-2/HAP group than in the other groups. At 8 weeks, the difference in new 
bone volume between the HAP only and ErhBMP-2/HAP groups was not significant 
(Table 5).
# Table 1 Total augmented area of each group (histomorphometric analysis)

|                      | Total augmented area (mm$^2$) | 2 weeks  | 8 weeks  |
|----------------------|-------------------------------|----------|----------|
| Control (n = 10)     | 6.34 ± 0.17                   | 6.54 ± 0.32 |
| HAP (n = 5)          | 9.51 ± 0.61*                  | 8.64 ± 0.38* |
| ErhBMP-2/HAP (n = 5) | 10.05 ± 0.52*                 | 9.02 ± 0.55* |

Values are means ± standard deviation; n = number of specimens.

* Significant difference compared with control group ($p < 0.05$).

# Table 2 New bone area of each group (histomorphometric analysis)

|                      | New bone area (mm$^2$) | 2 weeks  | 8 weeks  |
|----------------------|------------------------|----------|----------|
| Control (n = 10)     | 1.25 ± 0.09            | 1.39 ± 0.13 |
| HAP (n = 5)          | 2.94 ± 0.28*           | 3.68 ± 0.24* |
| ErhBMP-2/HAP (n = 5) | 4.75 ± 0.50*†          | 3.67 ± 0.19* |

Values are means ± standard deviation; n = number of specimens.

* Significant difference compared with control group ($p < 0.05$).

† Significant difference between HAP and ErhBMP-2/HAP groups ($p < 0.05$).

# Table 3 Residual particle area of each group (histomorphometric analysis)

|                      | Residual particle area (mm$^2$) | 2 weeks  | 8 weeks  |
|----------------------|---------------------------------|----------|----------|
| Control (n = 10)     | NA                              | NA       |
| HAP (n = 5)          | 1.99 ± 0.14                     | 1.91 ± 0.38 |
| ErhBMP-2/HAP (n = 5) | 1.90 ± 0.34                     | 1.82 ± 0.23 |

Values are means ± standard deviation; n = number of specimens.
### Table 4 Total augmented volume of each group (tomographic analysis)

| Total augmented volume (mm$^3$) | 2 weeks  | 8 weeks |
|---------------------------------|---------|---------|
| Control (n = 10)                | 5.78 ± 0.54 | 12.61 ± 1.16 |
| HAP (n = 5)                     | 56.31 ± 2.62* | 72.66 ± 4.07* |
| ErhBMP-2/HAP (n = 5)            | 61.12 ± 1.84* | 67.55 ± 5.48* |

Values are means ± standard deviation; n = number of specimens.

* Significant difference compared with control group (p < 0.05).

### Table 5 New bone volume of each group (tomographic analysis)

| New bone volume (mm$^3$) | 2 weeks  | 8 weeks |
|--------------------------|---------|---------|
| Control (n = 10)         | 5.78 ± 0.54 | 12.61 ± 1.16 |
| HAP (n = 5)              | 28.40 ± 2.05* | 40.60 ± 2.82* |
| ErhBMP-2/HAP (n = 5)     | 34.57 ± 1.65** | 38.31 ± 3.34* |

Values are means ± standard deviation; n = number of specimens.

* Significant difference compared with control group (p < 0.05).

† Significant difference between HAP and ErhBMP-2/HAP groups (p < 0.05).
IV. Discussion

BMP is a key factor in bone regeneration and healing. CHO BMP-2 is relatively expensive because of low production volumes. ErhBMP-2 is particularly attractive for biotechnology because of the ability of E. coli to grow rapidly and at high density on inexpensive substrates. Recombinant DNA techniques have been used to produce BMP-2 as an alternative to autograft bone to enhance healing of intraosseous defects.

Many studies have been performed to assess potential rhBMP-2 carriers. Either platelet-rich plasma or calcium phosphate can be used as a carrier of rhBMP-2 (Jiang et al., 2012; Schmidlin et al., 2013). The efficacy of an absorbable collagen sponge has also been demonstrated (Jung et al., 2011; Visser et al., 2009). The Infuse® system (Medtronic, Memphis, TN, USA) consists of rhBMP-2 on an absorbable collagen sponge carrier. OP-1® (Stryker Biotech, Kalamazoo, MI, USA) consists of rhBMP-7 and bovine collagen that has been reconstituted with saline to form a paste. However, collagen is not able to maintain space, which is crucial for excluding unwanted cells. For space maintenance during wound healing, biphasic calcium phosphate with a high proportion of HA may be a more appropriate rhBMP-2 carrier (Yun et al., 2014). Bioactive glass fabricated with dicalcium phosphate dehydrate is
not suitable as a BMP-2 carrier; a previous study showed that the bone mineral density, bone area, and bone mineral content of tibiae and contralateral femurs did not differ between control and BMP-treated groups (Liu et al., 2014). The goals of this study were to evaluate the effect of HAP on bone regeneration and to determine the efficacy of HAP as a carrier for ErhBMP-2 in a rabbit calvarial intraosseous defect model.

Collagen carriers do not resist collapse caused by soft tissue pressure during bone formation. An investigation of the bone cell response to titanium surfaces showed that bone cell activities were enhanced in the presence of a BMP–atelopeptide type I collagen mixture (Ong et al., 1999). Another study examined the effects of a BMP–atelopeptide type I collagen mixture on bond strength at the interface between bone and titanium implants. At 3 weeks post-surgery, the reverse torque of the BMP-treated group (74.2 ± 5.2 N·cm) was significantly greater than the reverse torque of the untreated group (32.8 ± 1.1 N·cm). At 12 weeks post-surgery, the difference between the reverse torque of the BMP-treated group (89.2 ± 2.7 N·cm) and that of the untreated group (75.8 ± 2.4 N·cm), although still statistically significant, was much smaller (Bessho et al., 1999). These results are concordant with the results of the present study, suggesting that the soaked carriers released the ErhBMP-2 early; this is a major limitation of currently available carriers.

Our histomorphometric analyses showed that the HAP only and ErhBMP-2/HAP
groups had significantly larger areas of new bone than the control group at 2 weeks post-surgery. The ErhBMP-2/HAP group also differed significantly in new bone area from the control and HAP groups. Surprisingly, although the ErhBMP-2/HAP group showed a larger area of new bone than the HAP only group at 2 weeks post-surgery, no significant difference in new bone area was observed between the ErhBMP-2/HAP and HAP only groups at 8 weeks post-surgery. Thus, it can be inferred that the use of ErhBMP-2 with HAP as the carrier promoted rapid initial bone regeneration. According to Zhu et al. (Zhu et al., 2010), the ability to repair bone defects decreases with time, although Nano-HA/rhBMP-2 composite artificial bone shows a good ability to repair bone defects.

Micro-CT images showed that the new bone volume of the ErhBMP-2/HAP group was significantly larger than that of the other groups at 2 weeks post-surgery. However, the ErhBMP-2/HAP and HAP only groups did not differ significantly in new bone volume at 8 weeks post-surgery. Consistent with this result, an in vitro study showed that in the initial period of cultivation and up to 72 hours, coating of HAP with type I collagen had positive effects on the viability and osteoblastic characteristics of osteoblastic cells (Turhani et al., 2007). Therefore, it can be deduced that when guided bone regeneration is clinically required, HAP soaked in ErhBMP-2 can be applied without a membrane, since HAP can promote rapid initial bone generation. This technique would be easier than guided bone regeneration using an
absorbable or non-absorbable membrane and could provide quick and easy promotion of rapid initial bone generation.

The total augmented area and volume did not differ between the HAP only and ErhBMP-2/HAP groups at 8 weeks post-surgery. In addition, the residual particle area did not differ significantly between 2 weeks and 8 weeks post-surgery. This indicates that HAP can maintain rigidity over a long period. The porous structure of HAP facilitated the infiltration and adherence of responsive cells, and the carrier itself became a component of the newly formed bone. HAP is osteoconductive and can maintain its original biocompatible form. Because of these qualities, HAP may be useful in augmentation of ridges and elevation of sinuses in clinical settings.

According to Lee et al. (Lee et al., 2010), there is a strong positive correlation between a high concentration of rhBMP and soft tissue swelling. It has been shown that the inflammatory response prompted by rhBMP lasts for only a short period. Although it varies according to volume, the degree of inflammation gradually decreases over the first 7 days; the authors therefore advise careful observation for 7 days after surgery. In our experiment, no side effects, such as seroma or edema, were observed for up to 8 weeks. This indicates that the amount of ErhBMP-2 used in this experiment was not high enough to cause serious side effects.

ErhBMP-2 is known to induce ectopic bone growth (Deutsch et al., 2010). If control and ErhBMP-treated bone defects are too close together, ErhBMP-2 might
flow into the control defect and cause unwanted bone regeneration, seroma, or edema (Tannoury et al., 2014). In this study, no specific evidence of complications was observed. In a previous study, doubts were raised over whether a distance of 2 mm was sufficient to prevent control calvarial defects from being affected by ErhBMP-2 in neighboring defects (Lee et al., 2014). In this study, a distance of 3 mm between the treated and control defects proved to be sufficient to allow comparison of healing responses. Therefore, ErhBMP-2-loaded HAP can be used as a graft material that does not affect nearby defects.

Although new bone volume in the ErhBMP-2/HAP group was rapidly promoted in the short term, it did not increase over the long term. This may be due to the limitations of HA as a carrier. According to Crouzier et al. (Crouzier et al., 2011), ErhBMP-2 adsorbed onto polyelectrolyte multilayer-coated films and, to a lesser extent, bare granules could be stored and remained bioactive for over 3 weeks. The in vivo release kinetics of BMP-2 from calcium-deficient hydroxyapatite (CDHA) scaffolds resembled the in vitro kinetics (Patel et al., 2008). Similar observations have been made in other ectopic and orthotopic animal models (Hernandez et al., 2012). Quantitative real-time PCR and enzyme-linked immunosorbent assay demonstrated that a lyophilized BMP-2/CDHA construct with trehalose (lyo-tre-BMP-2) significantly promoted osteogenic differentiation of bone marrow stromal cells (Zhao et al., 2013). The release rate of BMP-2 is critical to bone regeneration. BMP-2 was
nearly 100% released from lyo-tre-BMP-2 over 28 days. Adsorption of BMP-2 onto HA follows the Langmuir isotherm (Lu et al., 2015). HAP may have more adsorption sites for its high specific surface area than HA block bone. Therefore, HAP may provide more opportunities for binding of ErhBMP-2 molecules. To develop an effective carrier, a method to release ErhBMP-2 from HAP at a consistent rate is required. Once this problem is solved, long-term increases in the volume and area of bone regeneration are expected to be realized. In future work, we will attempt to develop a method for slow, consistent release of ErhBMP-2 during long-term healing. In addition, other carriers, such as β-TCP, should be analyzed and compared.
V. Conclusion

Combining ErhBMP-2 with HAP could significantly promote rapid initial new bone formation. Moreover, HAP graft could increase new bone formation and space maintenance, therefore it might be one of the effective carriers of ErhBMP-2. Furthermore, in the future, the identification of methods for slow and consistent release of ErhBMP-2 during long-term healing will be needed.
Reference

1. Ebara S, Nakayama K. Mechanism for the action of bone morphogenetic proteins and regulation of their activity. Spine. 2002;27:S10-5.

2. Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 1996;10:1580-94.

3. Wozney JM. Overview of bone morphogenetic proteins. Spine. 2002;27:S2-8.

4. Herford AS, Boyne PJ. Reconstruction of mandibular continuity defects with bone morphogenetic protein-2 (rhBMP-2). J Oral Maxillofac Surg. 2008;66:616-24.

5. Lan J, Wang ZF, Shi B, Xia HB. The influence of recombinant human BMP-2 on bone-implant osseointegration: biomechanical testing and histomorphometric analysis. Int J Oral Maxillofac Surg. 2007;36:345-9.
6. Ono M, Sonoyama W, Yamamoto K. Efficient bone formation in a swine socket lift model using Escherichia coli-derived recombinant human bone morphogenetic protein-2 adsorbed in beta-tricalcium phosphate. Cells Tissues Organs. 2014;199:249-55.

7. Bessho K, Konishi Y, Kaihara S. Bone induction by Escherichia coli-derived recombinant human bone morphogenetic protein-2 compared with Chinese hamster ovary cell-derived recombinant human bone morphogenetic protein-2. Br J Oral Maxillofac Surg. 2000;38:645-9.

8. Choi Y, Yun JH, Kim CS. Sinus augmentation using absorbable collagen sponge loaded with Escherichia coli-expressed recombinant human bone morphogenetic protein 2 in a standardized rabbit sinus model: a radiographic and histologic analysis. Clin Oral Implants Res. 2012;23:682-9.

9. Lee JK, Cho LR, Um HS. Bone formation and remodeling of three different dental implant surfaces with Escherichia coli-derived recombinant human bone morphogenetic protein 2 in a rabbit model. Int J Oral Maxillofac Implants. 2013;28:424-30.
10. Hong SJ, Kim CS, Han DK. The effect of a fibrin-fibronectin/beta-tricalcium phosphate/recombinant human bone morphogenetic protein-2 system on bone formation in rat calvarial defects. Biomaterials. 2006;27:3810-6.

11. Hannink G, Arts JJ. Bioresorbability, porosity and mechanical strength of bone substitutes: what is optimal for bone regeneration? Injury. 2011;42 Suppl 2:S22-5.

12. Annis P, Brodke DS, Spiker WR. The fate of L5-S1 with low dose BMP-2 and pelvic fixation, with or without interbody fusion, in adult deformity surgery. Spine. 2015. doi:10.1097/BRS.0000000000000867.

13. Yoshida K, Bessho K, Fujimura K. Enhancement by recombinant human bone morphogenetic protein-2 of bone formation by means of porous hydroxyapatite in mandibular bone defects. J Dent Res. 1999;78:1505-10.

14. Jensen SS, Broggini N, Hjorting-Hansen E. Bone healing and graft resorption of autograft, anorganic bovine bone and beta-tricalcium phosphate. A histologic and histomorphometric study in the mandibles of minipigs. Clin Oral Implants Res. 2006;17:237-43.
15. Guda T, Darr A, Silliman DT. Methods to analyze bone regenerative response to different rhBMP-2 doses in rabbit craniofacial defects. Tissue Eng Part C Methods. 2014;20:749-60.

16. Jiang ZQ, Liu HY, Zhang LP. Repair of calvarial defects in rabbits with platelet-rich plasma as the scaffold for carrying bone marrow stromal cells. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012;113:327-33.

17. Schmidlin PR, Nicholls F, Kruse A. Evaluation of moldable, in situ hardening calcium phosphate bone graft substitutes. Clin Oral Implants Res. 2013;24:149-57.

18. Jung JH, Yun JH, Um YJ. Bone formation of Escherichia coli expressed rhBMP-2 on absorbable collagen block in rat calvarial defects. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;111:298-305.

19. Visser R, Arrabal PM, Becerra J. The effect of an rhBMP-2 absorbable collagen sponge-targeted system on bone formation in vivo. Biomaterials. 2009;30:2032-7.

20. Yun PY, Kim YK, Jeong KI. Influence of bone morphogenetic protein and proportion of hydroxyapatite on new bone formation in biphasic calcium phosphate
graft: two pilot studies in animal bony defect model. J Craniomaxillofac Surg. 2014;42:1909-17.

21. Liu WC, Robu IS, Patel R. The effects of 3D bioactive glass scaffolds and BMP-2 on bone formation in rat femoral critical size defects and adjacent bones. Biomed Mater. 2014;9:045013.

22. Ong JL, Bess EG, Bessho K. Osteoblast progenitor cell responses to characterized titanium surfaces in the presence of bone morphogenetic protein-atelopeptide type I collagen in vitro. J Oral Implantol. 1999;25:95-100.

23. Bessho K, Carnes DL, Cavin R. BMP stimulation of bone response adjacent to titanium implants in vivo. COIR. 1999;10:212-8.

24. Zhu W, Wang D, Zhang X. Experimental study of nano-hydroxyapatite/recombinant human bone morphogenetic protein-2 composite artificial bone. Artif Cells Blood Substit Immobil Biotechnol. 2010;38:150-6.

25. Turhani D, Weissenbock M, Stein E. Exogenous recombinant human BMP-2 has little initial effects on human osteoblastic cells cultured on collagen type I.
coated/noncoated hydroxyapatite ceramic granules. J Oral Maxillofac Surg. 2007;65:485-93.

26. Lee KB, Taghavi CE, Song KJ. Inflammatory characteristics of rhBMP-2 in vitro and in an in vivo rodent model. Spine. 2011;36:E149-54.

27. Deutsch H. High-dose bone morphogenetic protein-induced ectopic abdomen bone growth. Spine J. 2010;10:e1-4.

28. Tannoury CA, An HS. Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery. Spine J. 2014;14:552-9.

29. Lee JW, Lim HC, Lee EU. 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. J Periodontal Implant Sci. 2014;44:178-83.

30. Crouzier T, Sailhan F, Becquart P. The performance of BMP-2 loaded TCP/HAP porous ceramics with a polyelectrolyte multilayer film coating. Biomaterials. 2011;32:7543-54.
31. Patel ZS, Ueda H, Yamamoto M. In vitro and in vivo release of vascular endothelial growth factor from gelatin microparticles and biodegradable composite scaffolds. Pharm Res. 2008;25:2370-8.

32. Hernandez A, Sanchez E, Soriano I. Material-related effects of BMP-2 delivery systems on bone regeneration. Acta Biomater. 2012;8:781-91.

33. Zhao J, Wang S, Bao J. Trehalose maintains bioactivity and promotes sustained release of BMP-2 from lyophilized CDHA scaffolds for enhanced osteogenesis in vitro and in vivo. PLoS One. 2013;8:e54645.

34. Lu Z, Huangfu C, Wang Y. Kinetics and thermodynamics studies on the BMP-2 adsorption onto hydroxyapatite surface with different multi-morphological features. Mater Sci Eng C Mater Biol Appl. 2015;52:251-8.
Figure Legends

Figure 1. Two circular intraosseous defects of 8 mm diameter were made in each rabbit calvarium. The experimental graft materials (A, ErhBMP-2/HAP; B, HAP only) were grafted into one defect, and the other defect was left untreated as a control.

Figure 2. Representative photomicrographs obtained at 2 weeks postoperation. (A) Control, (B) HAP only, (C) ErhBMP-2/HAP (hematoxylin and eosin, ×40, Arrowheads = defect margin)

Figure 3 Representative photomicrographs obtained at 8 weeks postoperation. (A) Control, (B) HAP only, (C) ErhBMP-2/HAP (hematoxylin and eosin, ×40, Arrowheads = defect margin)

Figure 4 Representative coronally sectioned micro-computed tomography images at 2 weeks postoperation. (A) ErhBMP-2/HAP group, (B) HA only group.

Figure 5 Representative coronally sectioned micro-computed tomography images at 8 weeks postoperation. (A) ErhBMP-2/HAP group, (B) HA only group.

Figure 6 Graphs showing histomorphometric analysis of total augmented area and new bone area (mm$^2$). (A) Two weeks postoperation, (B) 8 weeks postoperation. (NV, new bone volume; TV, total bone volume.)
Figure 7 Graphs showing tomographic analysis of total augmented volume and new bone volume (mm$^3$). (A) Two weeks postoperation, (B) 8 weeks postoperation. (NV, new bone volume; TV, total bone volume.)
FIGURES

Figure 1.
Figure 2.
Figure 3
Figure 4
Figure 6

A

2 weeks postoperation

B

8 weeks postoperation

Figure 6
Figure 7

A

|       | New bone volume | Total augmented volume |
|-------|-----------------|------------------------|
| Control | 30              | 50                     |
| HAP    | 50              | 70                     |
| ErhBMP-2/HAP | 70          | 90                     |

Bone volume (mm$^3$)

2 weeks postoperation

B

|       | New bone volume | Total augmented volume |
|-------|-----------------|------------------------|
| Control | 10              | 30                     |
| HAP    | 30              | 50                     |
| ErhBMP-2/HAP | 50          | 70                     |

Bone volume (mm$^3$)

8 weeks postoperation
국문요약

토끼 두개골 결손모형에 *Escherichia coli*로부터 유래된 제2형 재조합 인간 골형성 단백질을 수산화인회석 전달체에 적용한 조기 골재생 효과

통계교수 최성호
연세대학교 대학원 치의학과
정정훈

골형성 단백질은 인체 내 골전구세포와 간엽 세포, 골아 세포, 골외조직, 연골 세포에 분포되어 있다. 모든 골형성 단백질 가운데 제2형 골형성 단백질은 중간엽세포로부터 골세포의 모든 분화과정에서 주요한 역할을 한다. 골형성 단백질은 햄스터의 난소세포에서 얻는 방법과, *E. coli*를 이용하여 유전자 재조합으로 얻는 방법이 있다. *E. coli*를 통해 얻는 골형성 단백질이 많은 양을 생산할 수 있어서 상대적으로 가격이
저렴하지만 골재생의 효과는 두 골형성단백질 모두 비슷한 것으로 알려져 있다. 수산화인회석입자 안전하면서도 골형성 단백질에 친화력이 높은 전달체이다. 또한 수산화인회석입자는 느리게 흡수되거나 이식된 공간을 유지하는 지지대역할을 하면서 신생골을 형성한다. 따라서 이 연구의 목적인 수산화인회석입자의 골재생능력과 E-coli로부터 유래된 제2형 재조합 인간 골형성 단백질을 수산화인회석입자에 적용하여 전달체로서 수산화인회석의 가능성을 평가하고자 한다.

20마리의 실험 동물은 뉴질랜드 화이트 레빗을 사용하였고 연구 프로토콜은 연세대학교 동물 실험 윤리 규정을 준수하였다. 20마리의 웰성 백서에 8mm 지름을 갖는 임계크기의 두개부 결손을 한 마리당 2부위씩 3mm의 거리를 두고 형성하였다. 결손부를 무작위로 나누어 한 군은 수산화인회석입자만 이식한 군, 다른 한군은 E-coli로부터 유래된 제2형 재조합 단백질을 적용한 수산화인회석입자군으로 분류하였다. 각 결손부에는 아무것도 이식하지 않은 sham surgery군을 대조군으로 두었다. 0.1mg E-coli로부터 유래된 제2형 재조합 단백질을 0.15ml를 0.1g 수산화인회석입자에 10분동안 적시어 1.5ug 용량의 E-coli로부터 유래된 제2형 재조합
단백질이 수산화인화석입자와 결합할 시간을 주었다. 각 군을술 후 2주와 8주에 희생하여 치유 결과를 임상적, 조직학적, 조직계측학적, 단층촬영 분석으로 비교 관찰하였다.

임상적으로 실험 후 두 치유기간동안 염증, 부종, 알레르기 반응 등의 부작용은 나타나지 않았다. 2주 치유기간에서 조직학적분석시 E. coli로부터 유래된 제 2형 재조합 단백질을 적용한 수산화인화석입자군의 신생골형성이 결손경계부위에서 두드러짐을 관찰하였다. 2주 치유기간에서 조직계측학적분석과 단층촬영분석시 new bone area와 new bone volume이 E. coli로부터 유래된 제 2형 재조합 단백질을 적용한 수산화인화석입자군과 수산화인화석입자만 이식한 군에서 대조군보다 유의하게 증가하였고, E. coli로부터 유래된 제 2형 재조합 단백질을 적용한 수산화인화석입자군에서 수산화인화석입자만 이식한 군보다 유의하게 증가하였다. 그러나 8주 치유기간에서 new bone area와 new bone volume이 E. coli로부터 유래된 제 2형 재조합 단백질을 적용한 수산화인화석입자군과 수산화인화석입자만 이식한 군간에 유의한 차이가 없었고, 두 군 모두 대조군에 비해서는 유의한 증가를 보였다. Total augmented area와 volume은 2주와 8주에서 E. coli로부터 유래된 제 2형
재조합 단백질을 적용한 수산화인회석입자군과 수산화인회석입자만 이식한 군간 유의한 차이가 없었다.

_E-coli_로부터 유래된 제 2형 재조합 단백질이 수산화인회석에 흡수되는 것은 밍큐어 흡착 등은식을 따른다. 본 실험에서 큰 수산화인회석 골편을 사용하지 않고 작은 수산화인회석입자를 사용하였다. 수산화인회석입자는 _E-coli_로부터 유래된 제 2형 재조합 단백질이 결합하기 쉬운 다공성을 가져서 친화력을 높였고, _E-coli_로부터 유래된 제 2형 재조합 단백질이 수산화인회석에 결합할 기회가 늘어나 전달체로서 수산화인회석의 수용능력이 증가하였다고 볼 수 있다. 이상의 연구를 통해 _E-coli_로부터 유래된 제 2형 재조합 단백질을 적용한 수산화인회석입자는 골형성이 조기에 촉진된 것을 확인할 수 있었다. 더욱이 수산화인회석입자는 골형성이 이루어지기까지 공간유지능력을 가지고 있다. 따라서 수산화인회석입자는 _E-coli_로부터 유래된 제 2형 재조합 단백질의 유용한 전달체로 사용될 수 있는 가능성을 보인다.

핵심되는 말: _Escherichia coli_로부터 유래된 제 2형 재조합 단백질, 수산화인회석, 골재생, 조직공학, 백서 두개골 결손부

41