

The Effect of Recombinant Human Bone  
Morphogenetic Protein-4 Dose on Bone  
Formation in Rat Calvarial Defects

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The Effect of Recombinant Human Bone  
Morphogenetic Protein-4 Dose on Bone  
Formation in Rat Calvarial Defects

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Eun-Kyoung Pang

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This certifies that the dissertation thesis  
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## 감사의 글

본 논문이 완성되기까지 부족한 저를 항상 격려해 주시고 사랑과 관심으로 이끌어 주신 조규성 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 따뜻한 관심으로 지켜봐 주신 김종관 교수님, 채중규 교수님, 최성호 교수님, 김창성 교수님께 진심으로 감사 드립니다.

연구 내내 많은 도움을 주신 치주과 교실원 여러분, 특히 임세웅 선생님, 석현주 선생님께 고마움을 전합니다.

그리고, 늘 아낌 없는 사랑과 헌신적인 도움으로 든든하고 따뜻한 버팀목이 되어준 사랑하는 나의 남편과 항상 한없는 따뜻함이 되어준 사랑하는 아들 한별에게 진정으로 사랑과 고마움의 마음을 전합니다.

마지막으로, 믿음과 사랑으로 이해해 주시고 항상 곁에서 든든하게 후원해주신 시부모님과 부모님께 감사의 마음을 담아 이 논문을 드립니다.

모든 분들께 진심으로 감사 드립니다.

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저자 씀

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## Abstract

# **The Effect of Recombinant Human Bone Morphogenetic Protein-4 Dose on Bone Formation in Rat Calvarial Defects**

Bone morphogenetic proteins (BMPs) are being evaluated as potential candidates for periodontal and bone regenerative therapy. The objective of this study was to evaluate the effect of recombinant human bone morphogenetic protein-4 (rhBMP-4) dose on local bone formation in the rat calvarial defect model.

Calvarial, 8-mm  $\varnothing$ , critical-size osteotomy defects were created in 140 male Sprague-Dawley rats. Seven groups of 20 animals each received either rhBMP-4(2.5  $\mu$ g) in an absorbable collagen sponge (ACS) carrier, rhBMP-4(5  $\mu$ g)/ACS, rhBMP-4(2.5  $\mu$ g) in a  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) carrier, rhBMP-4(5  $\mu$ g)/ $\beta$ -TCP, ACS and  $\beta$ -TCP carrier controls, or a sham surgery control and were evaluated by histologic and histometric parameters following a 2- and 8-week healing interval (10 animals/group/healing interval).

Surgical implantation of rhBMP-4/ACS and rhBMP-4/ $\beta$ -TCP resulted in enhanced local bone formation at both 2 and 8 weeks. Within the dose range examined, rhBMP-4 did not exhibit an appreciable dose dependent response. New bone area and defect closure were not significant different in rhBMP-4/ACS and rhBMP-4/ $\beta$ -TCP group. However, the bone densities of rhBMP-4/ACS group were

a significantly greater than those of the rhBMP-4/ $\beta$ -TCP group ( $P<0.01$ ). The augmented areas of the rhBMP-4/ $\beta$ -TCP group were significantly greater than those of the rhBMP-4/ACS group at 8 weeks( $P<0.01$ ).

In conclusion, rhBMP-4 combined with ACS or  $\beta$ -TCP has a significant potential to induce bone formation in the rat calvarial defect model. Within the selected rhBMP-4 dose range and observation interval, there appeared to be no meaningful differences in bone formation.

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**Key Words:** bone induction, recombinant human bone morphogenetic protein-4, dose response, absorbable collagen sponge,  $\beta$ -tricalcium phosphate, rat calvarial defect model

**The Effect of Recombinant Human Bone Morphogenetic  
Protein-4 Dose on Bone Formation  
in Rat Calvarial Defects**

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**I. Introduction**

Reconstruction of bone is often required in conjunction with dental and oral surgery procedures. Among the various procedures available to reconstruct bone, bone morphogenetic proteins (BMPs) are thought to constitute a promising technology. Since Urist first discovered BMPs (Urist, 1965), more than 20 BMPs have been identified, and several of these including BMP-2, -4, -5, -6, and -7 have been shown to be osteoinductive (Ahn et al., 2003; Choi et al., 2002; Gitelman et al., 1994; Kim et al., 2002; Sampath et al., 1992; Wikesjo et al., 1999). BMP-4 has been implicated as a coupling factor in bone turnover and appears to be involved in cellular events that precede callus formation during fracture repair. Moreover, BMP-4 exhibits a bone formation potential similar to that of BMP-2 in vitro (Kim et al., 2002) and in vivo (Ahn et al., 2003).

Previous studies have shown that the use of an appropriate carrier is critical for successful reconstruction of localized bone defects using BMPs(Kenley et al., 1994; Marden et al., 1994; Urist et al., 1984; Urist et al., 1987). An absorbable collagen sponge (ACS) has commonly been used as a carrier for BMPs(Choi et al., 2002; King et al., 1998). Although the ACS appeared an effective carrier in space-providing skeletal defects, it became victim to compressive forces when used for non-space-providing onlay indications(Barboza et al., 2000; Sigurdsson et al., 1997).  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) has been considered as an osteoconductive bone substitute and recently a biodegradable delivery system for BMPs(Ahn et al., 2003; Alam et al., 2001a; Alam et al., 2001b; Gao et al., 1996; Laffargue et al., 1999). Porous by nature,  $\beta$ -TCP has been shown to entrap rhBMP-4 within its micropores that the intrinsically diffusible rhBMP-4 may be retained and its action apparently prolonged(Urist et al., 1984). The porous structure of  $\beta$ -TCP allows cells to migrate into it, and, in addition, the biomaterial provides resistance against compressive forces.

It is likely that a critical minimal dose is required to support bone formation by BMPs. High doses may potentially produce adverse pharmacological effects, in addition to desired physiological effects. Increasing dosage may lead to increased vascularity, which in turn may cause excessive tissue edema(Marukawa et al., 2001). Therefore, it appears desirable to identify the lowest effective dose for the various BMP technologies and their potential indications. In our previous study using the

calvarial osteotomy model in the rat, 5µg rhBMP-4 in an ACS or β-TCP carrier proved sufficient to induce de novo bone formation(Ahn et al., 2003). In this study, we evaluated a rhBMP-4 dose of 2.5 µg in addition to the 5µg dose in this defect model in the rat.

## II. Materials & methods

### A. Materials

#### 1. Animals

A total of 140 male Sprague-Dawley rats (weight 200 - 300 g) were used. The animals were maintained in plastic cages in a room with 12 h-day/night cycles, an ambient temperature of 21°C, and *ad libitum* access to water and a standard laboratory pellet diet. Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

#### 2. rhBMP-4 Implants

rhBMP-4<sup>\*</sup> was reconstituted and diluted in buffer to produce a concentration of 0.025 or 0.05 mg/ml. A sterile 8-mm diameter ACS<sup>†</sup> or  $\beta$ -TCP<sup>‡</sup> particles were then loaded with 0.1 ml of the rhBMP-4 solutions to produce an implanted dose/defect of 2.5 and 5  $\mu$ g, respectively. For the control experiments, the buffer was used alone. The rhBMP-4 and control implants were fitted the calvarial defect following a 5-minute binding period.

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<sup>\*</sup> R&D Systems Inc., Minneapolis, MN, U.S.A

<sup>†</sup> Collatape<sup>®</sup>, Calcitek, Carlsbad, CA, U.S.A

<sup>‡</sup> Cerasorb<sup>®</sup>, 150-500  $\mu$ m, Curasan, Kleinotheim, Germany

## **B. Research Procedures**

### **1. Surgical procedures**

The animals were anaesthetized by an intramuscular injection (5 mg/kg body wt.) of a 4:1 solution of ketamine hydrochloride<sup>§</sup>: Xylazine<sup>||</sup>. Routine infiltration anaesthesia<sup>¶</sup> was used at the surgical site. An incision was made in the sagittal plane across the cranium and a full thickness flap reflected, exposing the calvarial bone. A standardized, circular, transosseous defect, 8 mm in diameter, was created on the cranium with the use of a saline cooled trephine drill<sup>#</sup>. After removal of the trephined calvarial disk, rhBMP-4 and control treatments were applied to the defects. Seven groups of 20 animals each either received rhBMP-4(2.5 µg)/ACS, rhBMP-4(5 µg)/ACS, rhBMP-4(2.5 µg)/β-TCP, rhBMP-4(5 µg)/β-TCP, ACS or β-TCP carrier controls, or sham surgery control. The periosteum and skin were then closed and sutured with 4-0 coated Vicryl violet<sup>\*\*</sup>.

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<sup>§</sup> Ketalar<sup>®</sup>, Yuhan Co., Seoul, Korea

<sup>||</sup> Rompun<sup>®</sup>, Bayer Korea, Seoul, Korea

<sup>¶</sup> 2% lidocaine, 1:100,000 epinephrine, Kwangmyung Pharm., Seoul, Korea

<sup>#</sup> 3i, FL, USA

<sup>\*\*</sup> Polyglactin 910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, UK

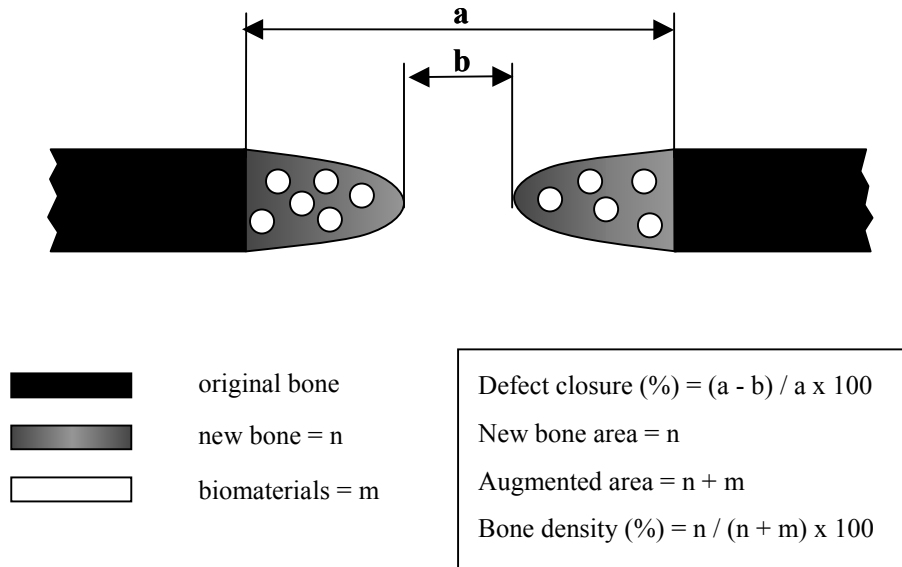
## **2. Histologic and histometric procedures**

The animals were sacrificed by CO<sub>2</sub> asphyxiation at 2 and 8 weeks postsurgery. Block sections including the surgical sites were removed. Samples were placed immediately into vials and were fixed in 10% neutral buffered formalin solution for 10 days. All samples were decalcified in EDTA-HCl for 7 days, and embedded in paraffin. Three µm thick coronal sections through the center of the circular defects were stained with hematoxylin-eosin. After conventional microscopic examination, computer-assisted histometric measurements of the newly formed bone were obtained using an automated image analysis system<sup>††</sup> coupled with a video camera on a light microscope<sup>‡‡</sup>. Sections were examined at 20x magnification. Defect closure was determined by measuring the distance between the defect margin and ingrowing bone margin. And it was expressed in mm and as a percentage of the total defect width. Augmented area (mm<sup>2</sup>) was measured including new bone, the residual biomaterials. New bone area (mm<sup>2</sup>) was determined by newly formed bone area within the augmented area, excluding biomaterials, marrow and fibrovascular tissues within the newly formed bone. Bone density was calculated as follows: Bone density (%) = New bone area / Augmented area x 100 (Figure 1).

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<sup>††</sup> Image-Pro Plus<sup>®</sup>, Media Cybernetics, Silver Spring, MD, U.S.A

<sup>‡‡</sup> Olympus BX50, Olympus Optical Co., Tokyo, Japan



**Figure 1.** Schematic drawings of calvarial osteotomy defect showing histometric analysis

### 3. Statistical Analysis

Histomorphometric recordings from the samples were used to calculate group means ( $\pm$ SD). A two-way analysis of variance was used to analyze the effect of time and experimental conditions. The post hoc Scheffé's test was used to analyze the difference between the groups ( $P < 0.05$ ).

### **III. Results**

#### **A. Clinical observations**

Wound healing was generally uneventful and appeared similar for all rhBMP-4 doses and the controls.

#### **B. Histologic observations**

*Sham surgery control:* At 2 and 8 weeks postsurgery, defects filled with thin loose connective tissue with a minimal amount of new bone formation originating from the defect margins were observed. The defect center had collapsed (Figure 1a).

*Carrier control groups:* In both the ACS and  $\beta$ -TCP group, the defects were filled with dense, fibrous connective tissue and limited new bone formation was observed at the defect margins at 8 weeks. The ACS appeared to be completely resorbed (Figure 1b) whereas residual  $\beta$ -TCP particles were still observed (Figure 1c).

*rhBMP-4/ACS groups:* Irrespective of dose, all defect sites exhibited marked bone formation, and were almost completely bridged with the new bone at 8 weeks. The consolidation of lamellar bone along the dural aspect was observed at 2 weeks postsurgery and at 8 weeks the appearance of the new bone was more lamellar than that observed at 2 weeks. At 2 weeks postsurgery, ACS fragments were observed embedded within the new bone without connective tissue intervention. No residual

ACS could be detected at 8 weeks. There was no apparent relationship between the degree of bone maturity, presence of residual ACS, and rhBMP-4 dose (Figure 2).

*rhBMP-4/ $\beta$ -TCP groups:* Regardless of dose, extensive bone regeneration was apparent in all defect sites. A large number of residual  $\beta$ -TCP particles were observed within the new bone at 2 weeks and appeared to be less at 8 weeks without apparent differences between rhBMP-4 doses. The appearance of the new bone at 8 weeks was more lamellar than that at 2 weeks, and did not appear to be correlated with the rhBMP-4 dose (Figure 3).

### **C. Histometric analysis**

Tables 1-4 show the results of the histometric analysis. Only limited new bone formation was observed in the sham surgery controls. New bone growth and defect closure in the ACS and  $\beta$ -TCP controls was not different from that in the sham surgery controls.

Irrespective of dose, there was a significant bone growth and the defects were almost completely closed in the rhBMP-4/ACS and rhBMP-4/ $\beta$ -TCP groups. New bone area and defect closure were not significant different between these two groups. In the bone density, rhBMP-4/ACS group had a significantly greater value than rhBMP-4/ $\beta$ -TCP group ( $P < 0.01$ ) and, there were no differences between the different dose level. The augmented areas of the rhBMP-4/ $\beta$ -TCP group were significantly

greater than those of the rhBMP-4/ACS group at 8 weeks( $P<0.01$ ).

The two-way ANOVA revealed that both time and treatment conditions significantly affected the formation of new bone within the defects ( $P<0.01$ ). There were statistically significant differences between the results obtained at 2 and 8 weeks in all groups ( $P<0.01$ ).

**Table 1. Defect closure (group means  $\pm$  SD; n=10, mm (%))**

	2 weeks	8 weeks
sham surgery control	0.9 $\pm$ 0.3 (13.9 $\pm$ 3.6)	1.2 $\pm$ 0.5 (15.6 $\pm$ 7.3)
ACS control	1.4 $\pm$ 0.5 (22.6 $\pm$ 9.3)	1.5 $\pm$ 0.5 (26.9 $\pm$ 10.4)
$\beta$ -TCP control	1.1 $\pm$ 0.4 (20.8 $\pm$ 7.2)	1.3 $\pm$ 0.6 (25.3 $\pm$ 10.8)
rhBMP-4(2.5 $\mu$ g)/ACS	6.7 $\pm$ 0.9 (96.9 $\pm$ 7.1) <sup>*†</sup>	6.8 $\pm$ 0.7 (99.6 $\pm$ 1.2) <sup>*†</sup>
rhBMP-4(5 $\mu$ g)/ACS	6.5 $\pm$ 0.5 (100.0 $\pm$ 0.0) <sup>*†</sup>	6.5 $\pm$ 0.7 (100.0 $\pm$ 0.0) <sup>*†</sup>
rhBMP-4(2.5 $\mu$ g)/ $\beta$ -TCP	6.7 $\pm$ 0.7 (98.4 $\pm$ 5.0) <sup>*‡</sup>	6.9 $\pm$ 0.7 (95.1 $\pm$ 10.1) <sup>*‡</sup>
rhBMP-4(5 $\mu$ g)/ $\beta$ -TCP	5.8 $\pm$ 1.0 (93.2 $\pm$ 12.6) <sup>*‡</sup>	6.5 $\pm$ 0.7 (100.0 $\pm$ 0.0) <sup>*‡</sup>

\*: Statistically significant difference compared to sham surgery control group (P<0.01)

†: Statistically significant difference compared to ACS control group (P<0.01)

‡: Statistically significant difference compared to  $\beta$ -TCP control group (P<0.01)

**Table 2. New bone area (group means  $\pm$  SD; n=10, mm<sup>2</sup>)**

	2 weeks	8 weeks
sham surgery control	0.3 $\pm$ 0.1	0.5 $\pm$ 0.2
ACS control	0.7 $\pm$ 0.2	0.9 $\pm$ 0.4
$\beta$ -TCP control	0.9 $\pm$ 0.3	1.2 $\pm$ 0.3
rhBMP-4(2.5 $\mu$ g)/ACS	3.8 $\pm$ 0.7 <sup>*†</sup>	4.9 $\pm$ 0.8 <sup>*†</sup>
rhBMP-4(5 $\mu$ g)/ACS	3.8 $\pm$ 0.7 <sup>*†</sup>	4.7 $\pm$ 0.8 <sup>*†</sup>
rhBMP-4(2.5 $\mu$ g)/ $\beta$ -TCP	4.3 $\pm$ 1.6 <sup>*‡</sup>	4.7 $\pm$ 1.7 <sup>*‡</sup>
rhBMP-4(5 $\mu$ g)/ $\beta$ -TCP	3.9 $\pm$ 0.9 <sup>*‡</sup>	4.5 $\pm$ 0.7 <sup>*‡</sup>

\*: Statistically significant difference compared to sham surgery control group (P<0.01)

†: Statistically significant difference compared to ACS control group (P<0.01)

‡: Statistically significant difference compared to  $\beta$ -TCP control group (P<0.01)

**Table 3. Bone density (group means  $\pm$  SD; n=10, %)**

	2 weeks	8 weeks
rhBMP-4(2.5 $\mu$ g)/ACS	62.4 $\pm$ 11.4	90.2 $\pm$ 5.4*
rhBMP-4(5 $\mu$ g)/ACS	68.7 $\pm$ 2.9 <sup>†</sup>	91.1 $\pm$ 2.5 <sup>†</sup>
rhBMP-4(2.5 $\mu$ g)/ $\beta$ -TCP	64.4 $\pm$ 4.6	69.7 $\pm$ 11.8
rhBMP-4(5 $\mu$ g)/ $\beta$ -TCP	61.8 $\pm$ 2.0	72.3 $\pm$ 2.8

\*: Statistically significant difference compared to 2.5  $\mu$ g rhBMP-4/ $\beta$ -TCP group at 8 weeks (P<0.05)

<sup>†</sup>: Statistically significant difference compared to 5  $\mu$ g rhBMP-4 / $\beta$ -TCP group (P<0.01)

**Table 4. Augmented area at 8 weeks postsurgery (group means  $\pm$  SD; n=10, mm<sup>2</sup>)**

	8 weeks
rhBMP-4(2.5 $\mu$ g)/ACS	5.3 $\pm$ 0.9
rhBMP-4(5 $\mu$ g)/ACS	5.3 $\pm$ 0.8
rhBMP-4(2.5 $\mu$ g)/ $\beta$ -TCP	7.0 $\pm$ 2.0*
rhBMP-4(5 $\mu$ g)/ $\beta$ -TCP	6.2 $\pm$ 0.8

\*: Statistically significant difference compared to 5  $\mu$ g rhBMP-4/ACS group (P<0.05)

## **IV. Discussion**

The objective of this study was to evaluate the effect of rhBMP-4 dose in an ACS or  $\beta$ -TCP carrier on bone regeneration in a critical size rat calvarial defect model. The experimental defects receiving rhBMP-4 at 2.5 and 5  $\mu$ g underwent extensive bone formation following a 2- and 8-week healing interval. The carrier and sham surgery controls exhibited limited, if any, evidence of new bone formation. Within the selected rhBMP-4 dose and observation interval, there appeared to be no significant differences in bone formation.

The experimental model used in this study was based on that described by Takagi and Urist(Takagi et al., 1982). This model has been shown effective to evaluate the potential for bone formation in many studies(Ahn et al., 2003). We selected this model for the following reasons: 1) rats were readily available; 2) the surgical procedures on the rat calvarial bone are relatively simple to perform; 3) spontaneous healing would not occur at the control site (critical size defect(Schmitz et al., 1986)); 4) the observations can be focused on the healing process of the bone, since there are no major nerves or blood vessels around the rat calvaria; 5) the calvarial defect model has many similarities to the maxillofacial region, as anatomically the calvaria consists of two cortical plates with a region of intervening cancellous bone similar to the mandible(Frame, 1980), and physiologically, the cortical bone in the calvaria resembles an atrophic mandible(Bays, 1983); 6) the preparation of the tissue

specimens is easy; and 7) the parameters can be simply and accurately measured in each specimen(Higuchi et al., 1999)

Dose-dependency may vary as a function of the carrier technology(Uludag et al., 1999; Winn et al., 1999), the species, the experimental site/application, the evolutionary status of the recipient(Winn et al., 1999), and the observation interval(Boden et al., 1998; Winn et al., 1999). In this study, we used 2.5 and 5  $\mu$ g rhBMP-4 for each defect in a rat calvarial defect model and a 2- and 8-week observation interval. Within this dose and observation interval in this model, it was apparent that rhBMP-4 did not produce an appreciable dose dependent response. In addition, there was no apparent relationship between the degree of remodeling or the presence of residual carrier biomaterial and rhBMP-4 dose. These findings are not unusual and corroborate data from several other studies. For example, Wikesjö et al.(1999) reported that canine supraalveolar periodontal defects treated with rhBMP-2 in ACS at 0.05, 0.10 or 0.20 mg/ml all exhibited extensive and similar alveolar bone formation(Wikesjo et al., 1999). Moreover, Tatakis et al.(2002) using the same rhBMP-2 concentrations also reported no meaningful differences in bone induction using supraalveolar peri-implant defects in dogs(Tatakis et al., 2002). Lastly, Zellin et al.(1999) placed dome-shaped barrier membranes made of expanded polytetrafluoroethylene on the parietal surface of rats and filled the domes with 5, 15  $\mu$ g of rhBMP-2 in ACS. They also reported that there was no correlation between bone formation within the domes and rhBMP-2 dose(Zellin et al., 1999).

Nevertheless some studies have shown a distinct dose dependent response(Alam et al., 2001a; Alam et al., 2001b; Kanatani et al., 1995; Kenley et al., 1994; Laffargue et al., 1999; Ohura et al., 1999; Wang et al., 1990), while still others have failed to demonstrate dose-dependency, even when wide dose ranges were used. The absence of a dose dependent response has been reported in both ectopic(Aspenberg et al., 1996; Winn et al., 1999; Zellin et al., 1999) and orthotopic (Cook et al., 1994; Sandhu et al., 1996; Tatakis et al., 2002; Wikesjo et al., 1999) applications in various species. In these *in vivo* studies, the lack of dose dependent response might be expected once a minimum dose threshold has been exceeded (Cook et al., 1994; Sandhu et al., 1996; Tatakis et al., 2002; Winn et al., 1999). Bone formation may not vary with an increasing rhBMP-4 dose above a minimum dose threshold. This suggestion is supported by data in previous study of ours(Kim et al., 2002). In an *in vitro* study, the expression of alkaline phosphatase(ALP) mRNA of mouse calvarial cells was increased in a dose-dependent fashion by rhBMP-4. However, at concentrations greater than 50 ng/ml, the expression of ALP mRNA did not increase but reached a plateau with increasing rhBMP-4 concentrations. Similar observations were reported in an *in vivo* study(Winn et al., 1999). A dose dependent response in bone formation was observed at 0, 10, or 50 µg rhBMP-2 using a poly(D,L-lactide) disk carrier technology in the rat ectopic model, however, at a dose of 100 µg rhBMP-2 bone formation did not further increase. In the present study, the 2.5 µg rhBMP-4 dose appears greater than the minimum dose threshold. To determine the

lowest effective rhBMP-4 dose in the rat calvarial model, additional studies using smaller doses appear necessary.

The regenerative potential of rhBMP-4 is known to depend upon the carrier technology. Many materials, such as collagen(Choi et al., 2002; Sigurdsson et al., 1996), demineralized bone matrix(Sigurdsson et al., 1996), hydroxyapatite(Mao et al., 1998), TCP(Gao et al., 1996; Urist et al., 1984; Urist et al., 1987; Wu et al., 1992), polylactic acid polymer(Heckman et al., 1991), polylactic-polyglycolic polymer(Miyamoto et al., 1993; Sigurdsson et al., 1996), gelatin(Isaksson et al., 1993), fibrin sealant(Kawamura et al., 1988), and composites of these materials(Ohura et al., 1999) have been used and evaluated as carriers of BMPs. In this study, we used ACS and  $\beta$ -TCP technologies as carriers for rhBMP-4. Animals receiving rhBMP-4/ $\beta$ -TCP exhibited greater total bone formation (area) compared to animals receiving rhBMP-4/ACS at 8 weeks. These results may be explained by the observation that ACS resorbed early and may have collapsed from soft tissue pressure imposed during the early healing events. In contrast, the  $\beta$ -TCP particles apparently exhibited sufficient firmness against the soft tissue compression to maintain the defect space. Also the slowly resorbing  $\beta$ -TCP biomaterial occupying the defect space may have displaced rhBMP-4 induced bone formation outside the defect area thus resulting in the more extensive bone formation. We used a  $\beta$ -TCP particle size of 150-500  $\mu\text{m}$ . It is not known whether the resorption rate of the  $\beta$ -TCP particles would increase with decreasing  $\beta$ -TCP particle size. Additional studies using  $\beta$ -TCP with

smaller particle sizes may be needed.

In conclusion, rhBMP-4 using ACS or  $\beta$ -TCP carrier technologies has significant potential to induce bone formation in rat calvarial critical size defects. Within the selected rhBMP-4 dose and observation interval, there appeared to be no meaningful differences in de novo bone formation. Both ACS and  $\beta$ -TCP may be considered effective carriers for rhBMP-4.

## **V. Conclusion**

The objective of this study was to evaluate the effect of recombinant human bone morphogenetic protein-4 (rhBMP-4) dose on local bone formation in the rat calvarial defect model.

Calvarial, 8-mm  $\varnothing$ , critical-size osteotomy defects were created in 140 male Sprague-Dawley rats. Seven groups of 20 animals each received either rhBMP-4(2.5  $\mu$ g) in an absorbable collagen sponge (ACS) carrier, rhBMP-4(5  $\mu$ g)/ACS, rhBMP-4(2.5  $\mu$ g) in a  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) carrier, rhBMP-4(5  $\mu$ g)/ $\beta$ -TCP, ACS and  $\beta$ -TCP carrier controls, or a sham-surgery control and were evaluated by histologic and histometric parameters following a 2- and 8-week healing interval (10 animals/group/healing interval).

Surgical implantation of rhBMP-4/ACS and rhBMP-4/ $\beta$ -TCP resulted in enhanced local bone formation at both 2 and 8 weeks. Within the dose range examined, rhBMP-4 did not exhibit an appreciable dose dependent response. New bone area and defect closure were not significant different in rhBMP-4/ACS and rhBMP-4/ $\beta$ -TCP group. However, the bone densities of rhBMP-4/ACS group were a significantly greater than those of the rhBMP-4/ $\beta$ -TCP group ( $P < 0.01$ ). The augmented areas of the rhBMP-4/ $\beta$ -TCP group were significantly greater than those of the rhBMP-4/ACS group at 8 weeks( $P < 0.01$ ).

rhBMP-4 combined with ACS or  $\beta$ -TCP has a significant potential to induce bone formation in the rat calvarial defect model. Within the selected rhBMP-4 dose range

and observation interval, there appeared to be no meaningful differences in bone formation.

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## Legends

**Figure 1.** Schematic drawings of osteotomy calvarial defect showing histometric analysis.

**Figure 2.** Representative photomicrographs of defect sites receiving the (a) sham-surgery control (b) ACS carrier control, and (c)  $\beta$ -TCP carrier control at 8 weeks postsurgery. Thin, fibrous connective tissues may be observed between the defect margins. The ACS biomaterial appears completely absorbed whereas residual  $\beta$ -TCP particles are still present within fibrous connective tissue at the defect site (asterisk =  $\beta$ -TCP, arrow head = defect margin; H-E stain; original magnification  $\times 20$ ).

**Figure 3.** Representative photomicrographs of defect sites receiving rhBMP-4(2.5  $\mu$ g)/ACS at 2 and 8 weeks postsurgery. At 2 weeks (a, b), some degraded ACS fragments were embedded within the new bone without connective tissue intervention, and there was some consolidation of lamellar bone along the dural aspect. At 8 weeks (c, d), the defect was almost completely filled with the new bone (arrow head = defect margin; H-E stain; original magnification a, c  $\times 20$ ; b, d  $\times 100$ ). Similar observations were made for defect sites receiving rhBMP-4(5  $\mu$ g)/ACS.

**Figure 4.** Representative photomicrographs of defect sites receiving rhBMP-4(2.5 $\mu$ g)/ $\beta$ -TCP at 2 and 8 weeks postsurgery. At 2 weeks (a, b), the defect was completely bridged with new bone, and a large number of residual  $\beta$ -TCP particles

were evident within the new bone. At 8 weeks (c, d), the  $\beta$ -TCP particles appeared smaller in numbers than at 2 weeks (asterisk =  $\beta$ -TCP, arrow head = defect margin; H-E stain; original magnification a, c  $\times 20$ ; b, d  $\times 100$ ). Similar observations were made for defect sites receiving rhBMP-4(5  $\mu$ g)/ $\beta$ -TCP.

## Figures ( I )

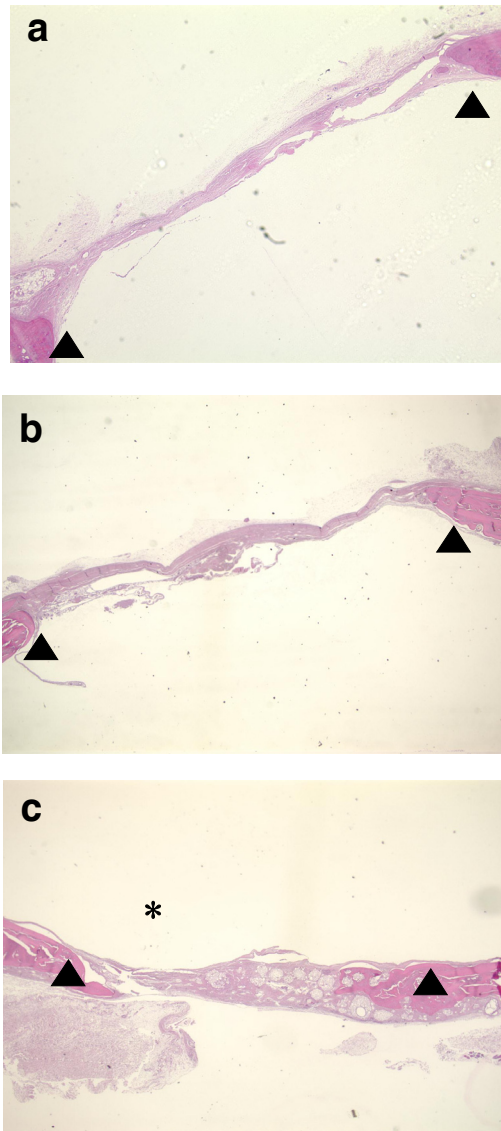


Figure 2

## Figures (II)

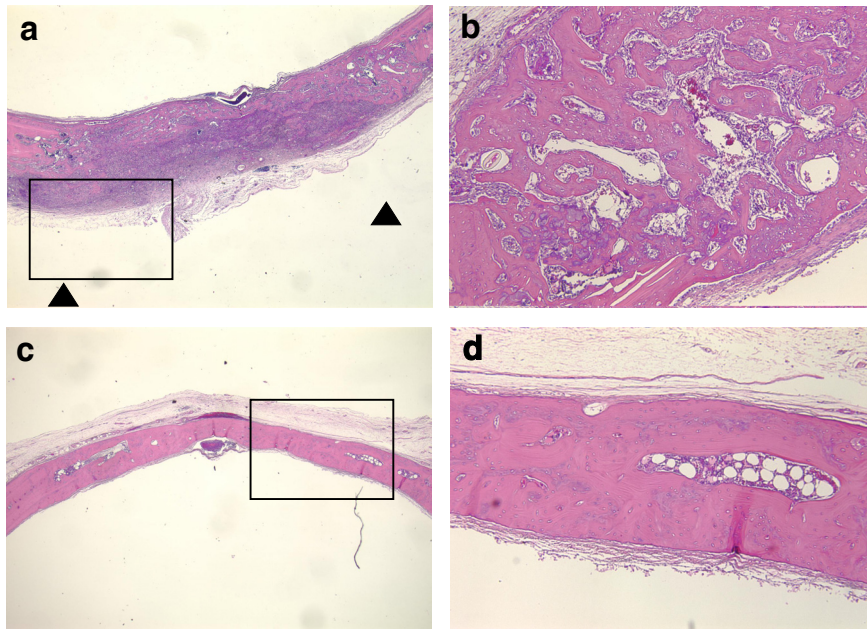


Figure 3

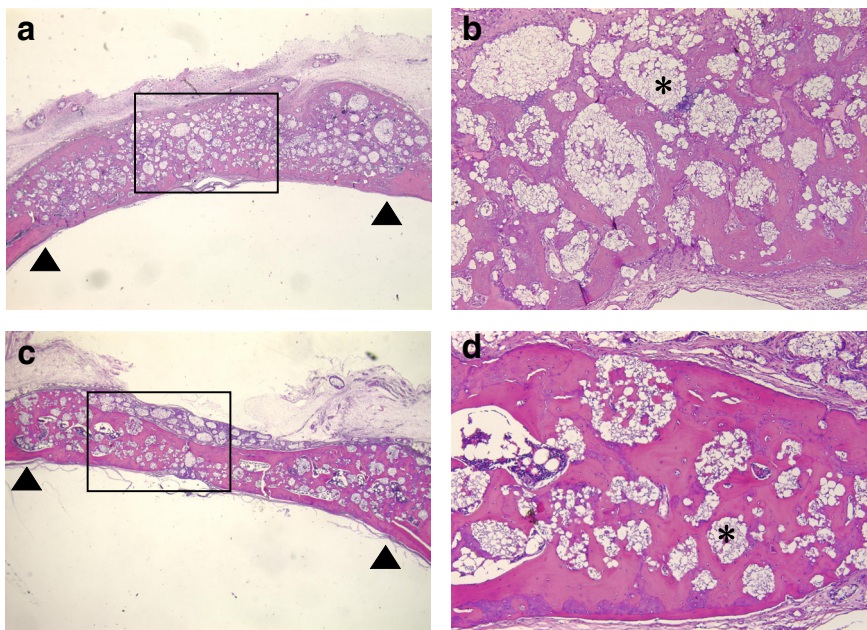


Figure 4

## 국문요약

# 백서 두개골 결손부에서 rhBMP-4의 용량에 따른 골재생 효과

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방 은 경

골형성 유도 단백질 (bone morphogenetic protein, BMP)은 치주 치료와 골 재생 치료를 위한 골 대체 물질의 하나로 연구, 평가되어왔다. 부작용 없이 재생효과를 최대화 하기 위해서는 BMP의 유효용량을 최소로 할 필요가 있다. 따라서 본 연구에서는 백서 두개골 결손부에서 rhBMP-4의 농도에 따른 골형성 유도 효과를 평가하고자 한다. 140마리의 웅성 백서에 지름 8 mm 임계크기 백서 두개골 결손부를 형성하였다. 동물은 각 20마리씩 rhBMP-4(2.5  $\mu$ g)/absorbable collagen sponge (ACS), rhBMP-4(5  $\mu$ g)/ACS, rhBMP-4(2.5  $\mu$ g)/ $\beta$ -tricalcium phosphate ( $\beta$ -TCP), rhBMP-4(5  $\mu$ g)/ $\beta$ -TCP, ACS,  $\beta$ -TCP를 이식하고, 대조군에는 아무것도 이식하지 않았다. 술 후 2주, 8주에 실험 동물을 희생하고, 조직학적 및 조직계측학적으로 비교 관찰하였다.

조직학적 관찰 결과, rhBMP-4/ACS 군과 rhBMP-4/ $\beta$ -TCP군 모두 술 후 2주와 8주에 뚜렷한 골형성 유도 효과를 보였으며, rhBMP-4 용량 2.5  $\mu$ g과 5  $\mu$ g에서 용량에 따른 골재생의 차이를 보이지는 않았다.

조직계측학적 관찰 결과, 신생골형성량 (new bone area)과 결손부 폐쇄 (defect closure)는 rhBMP-4/ $\beta$ -TCP군과 rhBMP-4/ACS군에서 차이가 없었으나, 골밀도 (bone density)는 rhBMP-4/ACS군에서 rhBMP-4/ $\beta$ -TCP군에 비해 유의성 있게 크게 나타났으며( $P<0.01$ ), 총조직형성량 (augmented area)은 rhBMP-4/ $\beta$ -TCP군에서 rhBMP-4/ACS군에 비해 유의성 있게 크게 나타났다( $P<0.01$ ).

이상의 결과에서 볼 때, 백서 두개골 결손부에서 ACS와  $\beta$ -TCP를 운반체로 사용하여 rhBMP-4를 적용하였을 때 골재생에 유의한 효과를 보였으며, 사용된 농도 범위에서 rhBMP-4 농도에 따른 골재생 효과의 차이는 없다고 사료된다.

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**핵심되는 말:** 신생골 형성, 골형성 유도 단백질, 농도, 흡수성 콜라겐 스폰지(ACS), 베타삼산화인산칼슘( $\beta$ -TCP), 백서 두개골 결손부