Effect of Recombinant Human Bone Morphogenetic Protein- 4 with Carriers in Rat Calvarial Defects

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Effect of Recombinant Human Bone Morphogenetic Protein- 4 with Carriers in Rat Calvarial Defects

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감사의 글

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본 연구 내내 많은 도움을 아끼지 않은 노현수 선생님과 치주과 교실원 여러분, 특히 석헌주 선생님, 권석훈 선생님, 그리고 이용준 선생님께 고마움을 전합니다.

그리고, 믿음과 사랑으로 이해해 주신 시부모님께 감사의 마음을 전합니다.

항상 곁에서 든든하게 후원해주시고 진심으로 사랑을 베풀어주신 사랑하는 부모님과 늘 사랑과 힘이 되어준 사랑하는 큰오빠 가족, 그리고 힘들어 하지 않고 통계처리를 도와준 사랑하는 작은 오빠께 감사 드립니다.

마지막으로, 늘 아낌 없는 사랑과 헌신적인 도움으로 든든하고 따뜻한 버팀목이 되어준 사랑하는 나의 남편에게 진정으로 사랑과 고마움의 마음을 담아 이 논문을 드립니다.

모든 분께 진심으로 감사 드립니다.

2002년 6월

저자 씀

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Abstract

Effect of Recombinant Human Bone Morphogenetic Protein- 4 with Carriers in Rat Calvarial Defects

Bone morphogenetic proteins (BMPs) are being evaluated as a candidate for periodontal and bone regenerative therapy. However, the research of recombinant human bone morphogenetic protein-4 (rhBMP-4) has been insufficient to evaluate its capacity to enhance bone formation and its carrier system. The purpose of this study was to evaluate the bone regenerative effect of rhBMP-4 delivered with absorbable collagen sponge (ACS) or β-tricalcium phosphate (β-TCP). In addition, we compared the potential of β-TCP to that of ACS as a carrier system for rhBMP-4.

8 mm calvarial critical-sized defects were created in one hundred male Sprague-Dawley rats. The animals were divided into 5 groups of 20 animals each. The defects were treated with rhBMP-4(rhBMP-4 at 0.05 ㎎/㎖)/ACS, rhBMP-4/β-TCP, ACS alone, or β-TCP alone, or were left untreated for surgical control. The rats were sacrificed at 2 or 8 weeks postsugery, and the outcomes were evaluated radiodensitometrically, histologically, and histomorphometrically.

The results of radiodesitometric analysis were as follows; at 2 weeks postsurgery, mean radiodensity (±SD) for the rhBMP-4/ACS group, the

rhBMP-4/β-TCP group, the ACS alone group, the β-TCP alone group, and the surgical control group amounted to 55.0±7.2%, 56.7±7.3%, 37.9±7.0%, 39.5±5.1%, and 15.3±2.9%, respectively, and, 61.4±12.6%, 76.1±5.8%, 34.0±3.4%, 42.7±7.9%, and 17.2±3.7%, respectively, at 8 weeks. The rhBMP-4/ACS group and the rhBMP-4/β-TCP group were significantly different from the other groups ($P<0.01$) at both 2 and 8 weeks, and the rhBMP-4/β-TCP group had a significantly greater radiodensity than the rhBMP-4/ACS group at 8 weeks ($P<0.01$).

The histologic observations were as follows; in the rhBMP-4/ACS group, some degraded ACS fragments were embedded within the new bone at 2 weeks. At 8 weeks, the defect was almost completely filled with the new bone and remnant of ACS could not be detected. In the rhBMP-4/β-TCP group, a lot of residual β-TCP particles and woven bone were evident at the defect sites at 2 weeks. At 8 weeks, residual β-TCP particles were less, and woven bone was greater than at 2 weeks.

The results of histomorphometric analysis were as follows; at 2 weeks postsurgery, mean bone fill (±SD) for the rhBMP-4/ACS group, the rhBMP-4/β-TCP group, the ACS alone group, the β-TCP alone group, and the surgical control group amounted to 71.7±2.8%, 60.8±.2.0%, 13.6±2.3%, 15.3±.2.0%, and 4.8±0.1%, respectively, and, 91.9±2.6%, 75.9±2.3%, 17.4±2.5%, 20.2±3.0%, and 8.2±0.0%, respectively, at 8 weeks. The

rhBMP-4/ACS group and the rhBMP-4/β-TCP group were significantly different from the other groups ($P<0.01$) and the rhBMP-4/ACS group had a significantly greater bone fill than the rhBMP-4/β-TCP group at both 2 and 8 weeks ($P<0.05$).

In conclusion, the bone regenerative effect of rhBMP-4/ACS was superior to that of rhBMP-4/β-TCP in the rat calvarial critical-sized defect. Surgical implantation of rhBMP-4/ACS may be used to support bone regeneration in the rat calvarial critical sized defect without complication, also rhBMP-4/β-TCP may be able to regenerate bone in the rat calvarial critical sized defect without any side effects occurring. In addition, both ACS and β-TCP may be considered as effective carriers for rhBMP-4.

Key words : bone regeneration; rhBMP-4; absorbable collagen sponge;
β-tricalcium phosphate; rat calvarial defect model

Effect of Recombinant Human Bone Morphogenetic Protein- 4 with Carriers in Rat Calvarial Defects

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Ⅰ. Introduction

Reconstructive surgery is performed to regenerate congenital and acquired bone defects such as periodontitis, degenerative arthritis, and osteomyelitis. To aid in the healing of these bone defects, it is often necessary to fill them with osteoinductive materials. The preferred treatment for most bone defects is the autograft. If prepared and handled carefully, the autograft is a good osteoinductive material. However, although highly effective, the autograft subjects the patient to additional operation, additional pain, hematoma, thrombosis, risk of donor site infection, nerve and vessel damage, a prolonged hospital stay and there is a limited supply of donor site material for large bone defects[41]. The allograft, although osteogenic, is the most common alternative to the autograft, but often induces an immune response in the host. This results

in destruction of blood vessels in the graft, disease transmission, and subsequent nonunion[8]. Thus, it may be better to use alternative substances for bone replacement such as, growth factors, transforming growth factor-β (TGF-β), and bone morphogenetic protein (BMP). In particular, BMPs are a set of growth and differentiation factors acting on early osteoprogenitor cells so that they differentiate into mature osteoblasts[48]. Recently, some investigators used modern biotechnology to isolate, identify, clone, and express seven individual BMPs[52,57]. Recombinant human bone morphogenetic proteins (rhBMPs) corresponding to the native BMPs have been expressed and characterized. The mature regions of BMP-2 and BMP-4 are more than 90% homologous at the amino acid level. BMP-4 has been implicated as a coupling factor in bone turnover and, during fracture repair, this protein appears to be involved in the cellular events which precede callus formation at the fracture site. Specifically, rhBMP-2 has been tested in many studies. However, rhBMP-4 has not yet been studied.

In spite of there being good prospects for BMP applications, the gap between research and the clinical use of BMP still remains, due to the lack of accessible delivery systems with bioactivity. Therefore, carrier systems are essential for delivering BMP, in order for its osteoinductive effect to be achieved. Carrier systems for delivering BMP should be biocompatible and

biodegradable to minimize local tissue response and to be replaced by newly formed bone[3]. Absorbable collagen sponge (ACS) has been studied and used extensively as a carrier for BMP. However, the collagen carrier acted only as a temporary template for osteoinduction of BMP at an early stage of bone formation and it was inappropriate for the maintenance of bone defects, since the collagen carrier was very absorbable. This problem motivated us to undertake research into other potential carrier systems. Recently, β-tricalcium phosphate (β-TCP) has gained particular interest as a carrier for BMP, due to its multiporous structure, which was assumed to entrap BMP and thus protect it from diffusion.

The purpose of this study was to evaluate the bone regenerative effect of rhBMP-4 delivered with ACS or β-TCP in the rat calvarial defect model. In addition, we compared the potential of β-TCP to that of ACS as a carrier system for rhBMP-4.

Ⅱ. Materials & methods

A. Materials

1. Animals

A total of 100 male Sprague-Dawley rats (body weight 200-300g) were used in this study. They were maintained in plastic cages in a room with 12 h-day/night cycles and an ambient temperature of 21℃ and allowed free access to water *ad libitum* and standard laboratory pellets. 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 construct

The rhBMP-4 implants consisted of rhBMP-4* in buffer loaded onto ACS¶ or β-TCP†. rhBMP-4 was used at a concentration of 0.05mg/ml.

For the rhBMP-4/ACS implant, a sterile 8mm diameter ACS was loaded with 0.1ml of the rhBMP-4 solution. And for the rhBMP-4/β-TCP implant, sterile β-TCP particles were loaded with 0.1ml of the rhBMP-4 solution. Following a 5-minute binding time, the implant was prepared to fit the calvarial defect.

B. Research Procedures

1. Surgical protocol

The animals were generally anaesthetized with an intramuscular injection (5mg/kg body wt.) consisting of Ketamine hydrochloride‡. During surgery, routine infiltration anaesthesia§ was used at the surgical site.

2. Defect induction

The surgical site was shaved and scrubbed with iodine. An incision was made in the sagittal plane across the cranium. A full thickness flap including periosteum was reflected, exposing the calvarial bone. Then, a standardized, round, transosseous defect 8 mm in diameter was created similarly on the cranium with the use of a saline cooled trephine drill# in the same manner as described by Schmitz and Hollinger[42] .

3. Wound management

Using different carrier systems, the animals were divided into 5 groups of 20 animals each and allowed to heal for 2 or 8 weeks. The animals were treated with either rhBMP-4 in ACS (rhBMP-4/ACS), rhBMP-4 in β-TCP (rhBMP-4/β-TCP), ACS alone, or β-TCP alone, or were left untreated (surgical control). The periosteum and skin were sutured for total coverage with 4-0 coated Vicryl® violet^.

Summary of study design was described in Table 1.

Table 1. Study design

Experimental group	Treatment
rhBMP-4/ACS	absorbable collagen sponge loaded with rhBMP-4
rhBMP-4/β-TCP	β-tricalcium phosphate loaded with rhBMP-4
ACS alone	absorbable collagen sponge alone
β-TCP alone	β-tricalcium phosphate alone
surgical control	the defects were left untreated

* R&D Systems Inc., Minneapolis, MN

¶ Collatape®, Calcitek, Carlsbad, CA

† Cerasorb®, 150-500 ㎛, Curasan, Lindigstrasse, Kleinotheim

‡ Ketalar®, Yuhan Co., Seoul, Korea

§ 2% lidocaine, 1:100,000 epinephrine, Kwangmyung Pharm., Seoul, Korea

3i, FL, USA

^ Polyglactin 910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, UK

4. Radiographic procedures

The animals were sacrificed by CO_2 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 radiographed using a dental X-ray unit† with an exposure time 0.1 sec (70kVp, 7mA) and 31×41㎜ dental radiographic film‡. Each radiograph was digitized by computer scanner* and the defect radiodensity (%) was assessed by a computer program#.

5. Histological procedures

Following the radiographic procedures, all samples were decalcified in EDTA-HCl for 7 days, and embedded in paraffin. Three ㎛ thick coronal sections through the center of circular defects were stained with hematoxylin-eosin stain (H-E). After conventional microscopic examination, computer-assisted histomorphometric measurements of newly formed bone were obtained using an automated image analysis system^ coupled with a video camera on a light microscope¶. Sections were analyzed under 20x magnification. A digitizer was used to trace the defect outline versus new bone formation, and a percentage of bone fill was determined.

6. Statistical Analysis

Histomorphometric recordings and radiodensitometric recordings from the samples were used to calculate means and standard deviations. For the comparison between all groups at a same time point, one-way analysis of variance was used ($P<0.05$), and the t-test was used to analyze the difference between the groups ($P<0.05$). For the comparison between 2 and 8 weeks in a same group, statistical significance was determined by the t-test ($P<0.05$).

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† Helicodent MD, SIEMENS AG, Germany

‡ Kodak Insight, Eastman Kodak, Rochester, NY

* Photosmart S20, Hewlett Packard, USA

Brain3dsp, NohsDiaTech Co., Korea

^ Image-Pro Plus®, Media Cybernetics, Silver Spring, M.D., USA

¶ Olympus BX50, Olympus Optical co., Japan

Ⅲ. Results

A. Radiographic observations

1. Surgical control group

At 2 and 8 weeks postsurgery, the defect center was radiolucent and the defect margin was obvious. (Figure1)

2. ACS alone group

At 2 and 8 weeks postsurgery, the defect center was radiolucent and the defect margin was evident. However, the radiolucency of the defect center at 2 weeks was less than at 8 weeks. (Figure 2)

3. β-TCP alone group

The defect margin was apparent and many small radiopaque dots were detected in the defect at 2 weeks postsurgery. At 8 weeks, radiolucency was observed in the defect center. (Figure 3)

4. rhBMP-4/ACS group

At 2 and 8 weeks postsurgery, the defect margin was a little difficult to detect. The defect center was radiopaque. (Figure 4)

5. rhBMP-4/β-TCP group

The defect margin was a little difficult to detect and many small radiopaque dots were observed in the defect at 2 weeks. At 8 weeks, the defect center was radiopaque. Therefore, the defect margin could not be detected. (Figure 5)

B. Radiodensitometric observations

The results of the radiographic analysis are shown in Table 2. At 2 weeks postsurgery, mean radiodensity (±SD) for rhBMP-4/ACS, rhBMP-4/β-TCP, ACS alone, β-TCP alone, and the surgical control amounted to 55.0±7.2%, 56.7±7.3%, 37.9±7.0%, 39.5±5.1%, and 15.3±2.9%, respectively, with significant difference compared to all groups ($P<0.01$). At 8 weeks postsurgery, the corresponding values were 61.4±12.6%, 76.1±5.8%, 34.0±3.4%, 42.7±7.9%, and 17.2±3.7%, respectively, with significant difference compared to all groups ($P<0.01$). rhBMP-4/β-TCP was significantly different from rhBMP-4/ACS at 8 weeks ($P<0.01$).

Table 2. Radiodensity analysis (group means ± SD ; n=10, %)

	2weeks	8weeks
rhBMP-4/ACS	55.0±7.2*¶	61.4±12.6*¶‡
rhBMP-4/β-TCP	56.7±7.3*†	76.1±5.8*†
ACS alone	37.9±7.0*	34.0±3.4*
β -TCP alone	39.5±5.1*	42.7±7.9*
surgical control	15.3±2.9	17.2±3.7

*: Statistically significant difference compared to surgical control group (P<0.01)

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

†: Statistically significant difference compared to β-TCP control group (P<0.01)

‡: Statistically significant difference compared to rhBMP-4/β-TCP (P<0.01)

C. Histologic observations

1. Surgical control group

At 2 and 8 weeks postsurgery, thin, fibrous connective tissue was present at the defect site (Figure 6). Also, there was a minimal amount of new bone formation originating from the defect margins. The defect center was collapsed. Inflammatory cell infiltration was minimal at the defect site.

2. ACS alone group

At 2 weeks postsurgery, there was dense, fibrous connective tissue at the defect site and ACS was partially degraded though still present. A little new bone formation adjacent to defect margins, and an obvious host bone-to-new bone interface was evident. Inflammatory cell infiltration was not observed at the defect site (Figure 7). At 8 weeks, a similar pattern to the 2 weeks observations was observed. However, the ACS was completely degraded (Figure 8).

3. β-TCP alone group

At 2 weeks postsurgery, a large number of residual β-TCP particles were present within fibrous connective tissue at the defect site. In addition, some new bone formation, adjacent to defect margins, was observed, and a host bone-to-new bone interface was apparent. Inflammatory cell infiltration was

minimal at the defect site (Figure 9). At 8 weeks, the histological observations were similar to the 2 weeks observations. Compared with the 2 weeks observations, residual β-TCP particles were less in number, and the quantity of new bone was greater than that observed at 2 weeks (Figure 10).

4. rhBMP-4/ACS group

At 2 weeks postsurgery, marked bone regeneration, as well as a consolidation of lamellar bone along the dural aspect, was observed. The degradation of the ACS had advanced considerably without any significant adverse reaction, and some degraded ACS fragments were embedded within the new bone, without connective tissue intervention. The defect margins tended to be connected with new bone (Figure 11). At 8 weeks, the defect was almost completely filled with the new bone. The quantity of the new bone was greater than that at 2 weeks and the appearance of the new bone was more lamellar than that observed at 2 weeks. No remnants of ACS could be detected. Differentiation of bone marrow was observed within the new bone (Figure 12).

5. rhBMP-4/β-TCP group

At 2 weeks postsurgery, a large number of residual β-TCP particles were observed within the new bone at the defect site. Occasionally, β-TCP particles were surrounded by woven bone. The new bone was found not only in direct

contact with β-TCP particles but also throughout the defect. And the host bone-to-new bone interface was a little difficult to detect (Figure 13). At 8 weeks, residual β-TCP particles were less in number compared to the 2 weeks observations. The quantity of the new bone was greater than that at 2 weeks and the appearance of the new bone was more lamellar than that observed at 2 weeks (Figure 14).

D. Histomorphometric observations

The results of the histomorphometric analysis are shown in Table 3. At 2 weeks postsurgery, mean bone fill (±SD) for rhBMP-4/ACS, rhBMP-4/β-TCP, ACS alone, β-TCP alone, and the surgical control amounted to 71.7±2.8%, 60.8±.2.0%, 13.6±2.3%, 15.3±.2.0%, and 4.8±0.1%, respectively, with significant difference compared to all groups ($P<0.01$). At 8 weeks postsurgery, the corresponding values were 91.9±2.6%, 75.9±2.3%, 17.4±2.5%, 20.2±3.0%, and 8.2±0.0%, respectively, with significant difference compared to all groups ($P<0.01$). rhBMP-4/ACS had a significantly greater bone fill than rhBMP-4/β-TCP at both 2 and 8 weeks ($P<0.05$).

Table 3. Histomorphometric analysis of bone fill (group means ± SD; n=10, %)

	2weeks	8weeks
rhBMP-4/ACS	71.7±2.8*¶‡	91.9±2.6*¶‡
rhBMP-4/β-TCP	60.8±2.0*†	75.9±2.3*†
ACS alone	13.6±2.3*	17.4±2.5*
β-TCP alone	15.3±2.0*	20.2±3.0*
surgical control	4.8±0.1	8.2±0.0

*: Statistically significant difference compared to surgical control group (P<0.01)

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

†: Statistically significant difference compared to β-TCP control group (P<0.01)

‡: Statistically significant difference compared to rhBMP-4/β-TCP (P<0.05)

Ⅳ. Discussion

The objective of this study was to evaluate the bone regenerative effect of recombinant human bone morphogenetic protein-4 (rhBMP-4) delivered with absorbable collagen sponge (ACS) or β-tricalcium phosphate (β-TCP). In addition, we compared the bone regenerative effect of rhBMP-4 delivered with β-TCP to that of ACS after the same length of time. We used an 8 mm calvarial critical sized defect model in the rat and rhBMP-4 was used at a concentration of 0.05mg/ml.

In this study, we used a so-called "critical sized defect", which implies that the defect does not heal by itself during the lifetime of the animal. In addition, we used calvarial defects that had previously been proved to be a good model for investigating the effects of BMPs in terms of bone induction. The rat calvarial defect, compared with other experimental bone defects, is a convenient model for studying bone regenerative materials, because of its effective accessibility and the lack of any fixation requirements. In addition, defects are reproducible and native, and induced healing processes have been well characterized[30]. Also, 8 millimeter trephine calvarial defects in rats have been shown to be critical sized defects[6,42,43,45]. This fact was consistent with the results of our study, in terms of surgical control group.

BMPs were first found in partially purified form from rabbit and bovine

demineralized bone matrix by Urist[48] and were isolated by Urist and coworkers in the 1980s[32,50]. BMPs are a part of the TGF- β superfamily of molecules, and form a set of growth and differentiation factors, acting on early osteoprogenitor cells, so that they differentiate into mature osteoblasts, resulting in the formation of new bone and new cartilage when implanted in animals [11,12,14,35,38,51]. BMPs have been applied in several osseous preclinical animal studies[16,55,56]. However, implantation of BMP alone did not induce bone formation because BMP tended to diffuse from the site of implantation. A carrier was needed to deliver BMP at a rate that permitted osteoinduction of BMP to be evoked [46,47,53]. Such a carrier material should be biocompatible, plastic so as to easily fill defects with the desired shape, biodegradable to minimize local tissue response and to be replaced by newly formed bone, enable the sustained release of BMP, as well as having mechanical stability in bone defects[3,15,20,28,37,47,49,51].

Many materials, such as tricalcium phosphate[9,49,51,59], polylactic acid polymer[14], collagen[7,10,44,55], demineralized bone matrix[14,21,44], hydroxyapatite [17,24,29,36], gelatin[18], fibrin sealant[22], polylactic-polyglycolic polymer[4,26,31,44,58], and composites of these materials[19,37,40,60] have been used and evaluated as a BMP carrier for healing of bone defects. Some of the BMP carrier requirements have been satisfied by these materials, but a rapidly resorbable carrier that has the desirable mechanical and plastic properties has yet to be

developed. In addition, osteoconductive biomaterials have been also utilized as the BMP carrier, but given the broad range of materials used, combined with differences in preclinical model systems, and the differences in the carrier's affinity for the BMPs, it is difficult to compare the effectiveness of these different carriers and to identify a preferred carrier, as has been reported in previous studies [15,18,57].

In this study, we used ACS and βTCP as carrier systems for rhBMP-4. ACS has been studied and used as an effective carrier for rhBMP[13,23,33,39]. Murata et al. reported that the collagen matrix was stably placed on the skull and suitable as a substitute for rhBMP-2 in the adult rat skull[34]. Also, Okubo et al. reported the osteoinducing activity of recombinant human bone morphogenetic protein-2 at intramuscular and intraskeletal sites in rats[38]. In this study, we used absorbable collagen sponge which consisted of bovine deep flexor tendon and which could be filled with fluid.

In the present study, the rhBMP-4/ACS group at 2 weeks postsurgery showed that ACS was partially resorbed and new bone was formed by the osteoinductive activity of rhBMP-4. At 8 weeks, ACS was completely resorbed and the defect was almost completely filled with lamellar bone including bone marrow. This result indicated that rhBMP-4 induced new bone formation early on, and that ACS might act as a suitable carrier to promote osteogenesis. This finding was consistent with previous studies[13,23,33,34,38,39].

Also, we used pure-phase β-TCP (phase purity > 99% β-modification of tricalcium phosphate) as the other carrier for rhBMP-4 in this study. β-TCP has been studied as an osteoconductive bone substitute and a biodegradable delivery system for rhBMP[1,2,5,9,25,27,54,59]. In this study, rhBMP-4/ β-TCP induced the differentiation of cartilage and woven bone at 2 weeks postsurgery, and woven bone and lamellar bone at 8 weeks. This finding was similar to that reported in the study by Urist et al[49]. Porous by nature, β-TCP would entrap rhBMP-4 within its micropores, and, in this way, the intrinsically diffusible rhBMP-4 was retained and its action consequently prolonged.

In our histomorphometric analysis, there were statistically significant differences between the results obtained at 2 weeks and those obtained at 8 weeks in all groups. These results may be explained by the fact that new bone formation in the defects increased from 2 weeks to 8 weeks, because the carrier materials were almost completely resorbed at 8 weeks. There were statistically significant differences between the rhBMP-4/ACS group and the ACS group, and between the rhBMP-4/β-TCP group and the β-TCP group. These findings showed that rhBMP-4 induced new bone formation in the defects. Since β-TCP was less resorptive than ACS, there was a statistically significant difference between the rhBMP-4/ACS group and the rhBMP-4/β-TCP group. However, the bioresorption of the β-TCP produced favorable conditions for rhBMP-2-induced bone formation[1].We used, a β-TCP particle

size of 150-500 ㎛. Concerning this point, we wondered whether the resorption rate of the β-TCP particles would increase with decreasing β-TCP particle size.

On the other hand, there were no statistically significant differences between the results obtained at 2 weeks and those obtained at 8 weeks in the rhBMP-4/ACS group, the ACS group or the β-TCP group, in terms of radiomorphometric analysis. These results might be due to the radiopacity of the ACS or β-TCP particles, which were partially resorbed and still present at 2 weeks. However there were statistically significant differences between the rhBMP-4/ACS group and the ACS group, and between the rhBMP-4/β-TCP group and the β-TCP group, at 2 and 8 weeks. These results showed that rhBMP-4 induced new bone formation in the defects. Also, there was a statistically significant difference between the rhBMP-4/ACS group and the rhBMP-4/β-TCP group at 8 weeks. In histological observations, ACS was almost completely resorbed, whereas β-TCP particles were still present at the defect sites at 8 weeks. Therefore, the combination of the radiodensity of the new bone induced by rhBMP-4 and the radiodensity of the β-TCP particles resulted in there being a greater radiodensity in the rhBMP-4/β-TCP group.

In conclusion, the bone regenerative effect of rhBMP-4/ACS was superior to that of rhBMP-4/β-TCP in the rat calvarial critical-sized defect. Surgical implantation of rhBMP-4/ACS may be used to support bone regeneration in

the rat calvarial critical sized defect without complication, also rhBMP-4/β-TCP may be able to regenerate bone in the rat calvarial critical sized defect, without any side effects occurring. In addition, both ACS and β-TCP may be considered as effective carriers for rhBMP-4. These findings may be applicable to the use of growth-stimulatory factors other than rhBMP-4 with biodegradable osteoconductive carrier materials, since these may offer additional clinical uses, for example, dental implants, alveolar ridge augmentation, and other conservative treatments. However, the calvarial defect in the rat, which we used, was relatively shallow, as regards depth of the defect. This may be a limiting factor, in terms of application of the results derived from the above model to the case of the clinical large bone defect. Therefore, further investigations are required using the clinical large bone defect model. On the other hand, the question as to whether β-TCP acts as a slow-release delivery system, potentiates the activity of BMP, or serves to distribute BMP in a more favorable manner, merits further investigation.

Ⅴ. Conclusion

The purpose of this study was to evaluate the bone regenerative effect of rhBMP-4 delivered with ACS or β-TCP in rat 8 mm calvarial defect model. In addition, we compared the potential of β- TCP to that of ACS as a carrier system for rhBMP-4. The male Sprague-Dawley rats were treated with either rhBMP-4 in ACS (rhBMP-4/ACS), rhBMP-4 in β- TCP (rhBMP-4/β-TCP), ACS alone, or β-TCP alone, or were left untreated (surgical control). The animals were sacrificed at 2 or 8 weeks postsugery, and the specimens were evaluated radiodensitometrically, histologically, and histomorphometrically.

The results of radiodesitometric analysis were as follows; the rhBMP-4/ACS group and the rhBMP-4/β-TCP group were significantly different from the other groups ($P<0.01$) at both 2 and 8 weeks, and the rhBMP-4/β-TCP group had a significantly greater radiodensity than the rhBMP-4/ACS group at 8 weeks ($P<0.01$).

The histologic observations were as follows;in the rhBMP-4/ACS group, and in the rhBMP-4/β-TCP group, the new bone was evident in the defect sites at 2 weeks and 8 weeks.The results of histomorphometric analysis were as follows; the rhBMP-4/ACS group and the rhBMP-4/β-TCP group were significantly different from the other groups ($P<0.01$), and the rhBMP-4/ACS group had a significantly greater bone fill than the rhBMP-4/β-TCP group at both 2 and 8 weeks ($P<0.05$).In conclusion, the bone regenerative effect of

rhBMP-4/ACS was greater than that of rhBMP-4/β-TCP. Surgical implantation of rhBMP-4/ACS may be used to support bone regeneration in the rat calvarial critical sized defect without complication.Also, rhBMP-4/β-TCP may be potent to regenerate bone in the rat calvarial critical sized defect without any side effect occurring. Therefore, both ACS and β-TCP may be considered as effective carriers for rhBMP-4.

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Legends

Figure 1. Representative radiographs of the surgical control group at 2 and 8 weeks postsurgery. The defect margin was evident.

Figure 2. Representative radiographs of the ACS alone group at 2 and 8 weeks postsurgery. The defect margin was obvious.

Figure 3. Representative radiographs of the β-TCP alone group at 2 and 8 weeks postsurgery. The defect margin was obvious and many small radiopaque dots were detected at the defect at 2 weeks.

Figure 4. Representative radiographs of the rhBMP-4/ACS group at 2 and 8 weeks postsurgery. The defect margin was a little difficult to detect.

Figure 5. Representative radiographs of the rhBMP-4/β-TCP group at 2 and 8 weeks postsurgery. The defect margin was a little difficult to detect and many small radiopaque dots were detected at the defect at 2 weeks.

Figure 6. Representative photomicrographs of the surgical control group at 2 and 8 weeks postsurgery. Thin, fibrous connective tissues between the defect margins were observed (H-E stain: an original magnification ×20). (arrow head: defect margin)

Figure 7. Representative photomicrographs of the ACS alone group at 2 weeks postsurgery. There were dense, fibrous connective tissues at the defect site and

ACS was partially degraded and still present (H-E stain: an original magnification ×20, ×100). (star: ACS, arrow head: defect margin)

Figure 8. Representative photomicrographs of the ACS alone group at 8 weeks postsurgery. The fibrous connective tissues were found at the defect and ACS was completely degraded (H-E stain: an original magnification ×20,×100). (arrow head: defect margin)

Figure 9. Representative photomicrographs of the β-TCP alone group at 2 weeks postsurgery. A large number of residual β-TCP particles were evident within fibrous connective tissue at the defect site (H-E stain: an original magnification ×20, ×100). (star: β-TCP, arrow head: defect margin)

Figure 10. Representative photomicrographs of the β-TCP alone group at 8 weeks postsurgery. The fibrous connective tissues appeared at the defect and β-TCP particles were resorbed (H-E stain: an original magnification ×20,×100). (star: β-TCP, arrow head: defect margin)

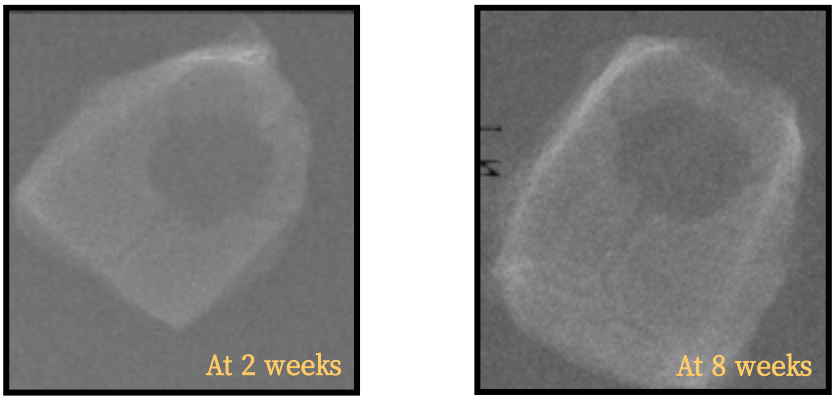
Figure 11. Representative photomicrographs of the rhBMP-4/ACS group at 2 weeks postsurgery. Some degraded ACS fragments were embedded within the new bone without connective tissue intervention (H-E stain: an original magnification ×20,×100). (star: ACS)

Figure 12. Representative photomicrographs of the rhBMP-4/ACS group at 8 weeks postsurgery. The defect was almost completely filled with the new bone (H-E stain: an original magnification ×20, ×100).

Figure 13. Representative photomicrographs of the rhBMP-4/β-TCP group at 2 weeks postsurgery. A large number of residual β-TCP particles were evident within the new bone at the defect site (H-E stain: an original magnification ×20, ×100). (star: β-TCP)

Figure 14. Representative photomicrographs of the rhBMP-4/β-TCP group at 8 weeks postsurgery. The β-TCP particles were resorbed and some β-TCP particles were surrounded by woven bone (H-E stain: an original magnification ×20, ×100). (star: β-TCP)

Figures (Ⅰ)



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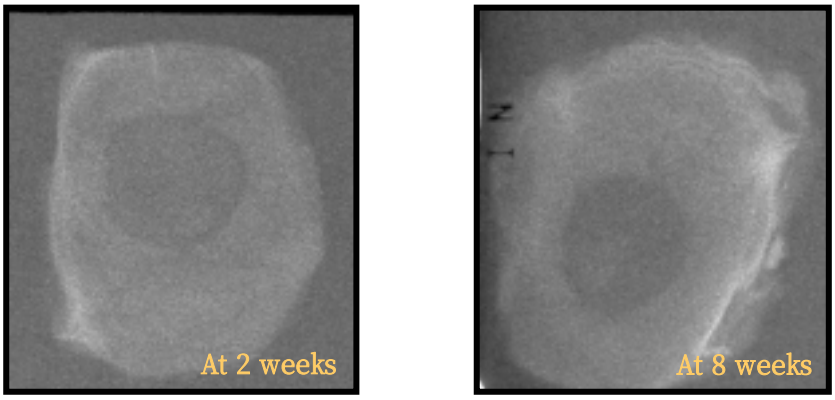

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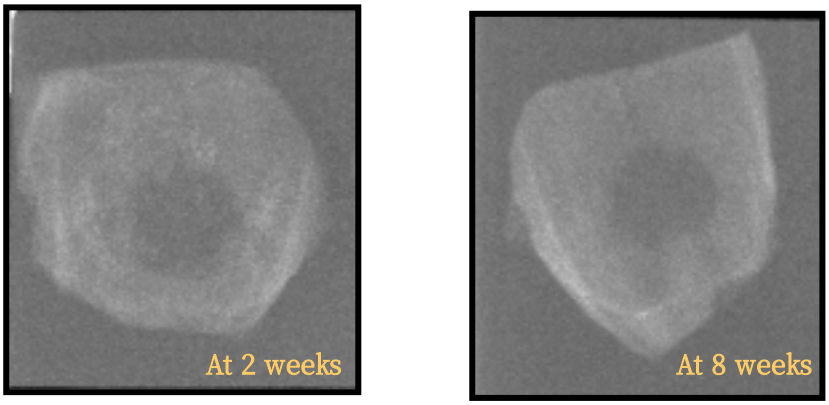

Figure 3.

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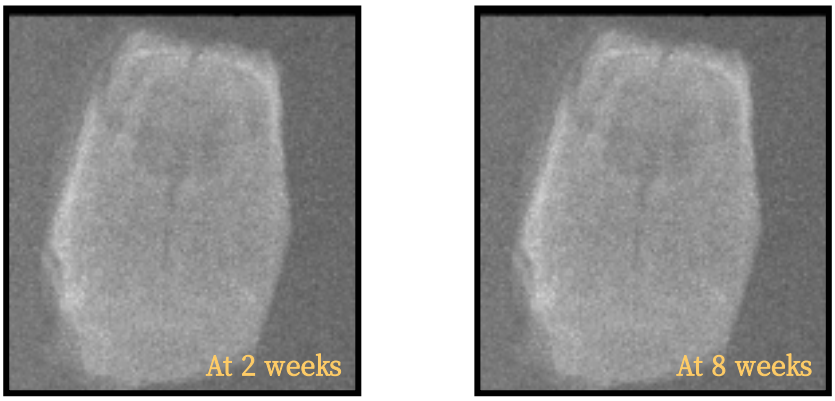

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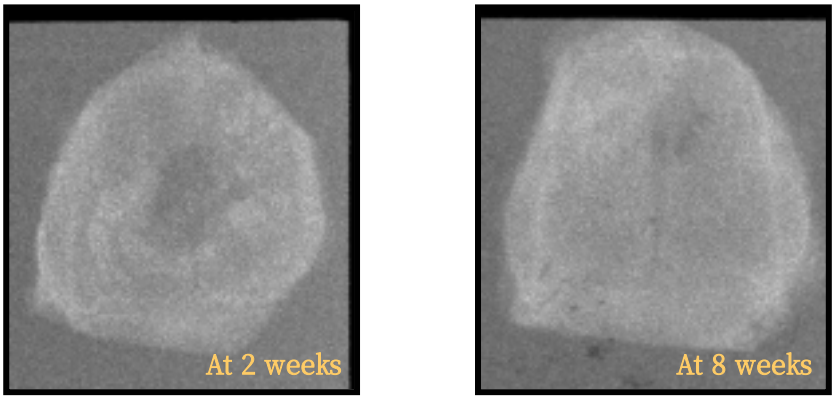

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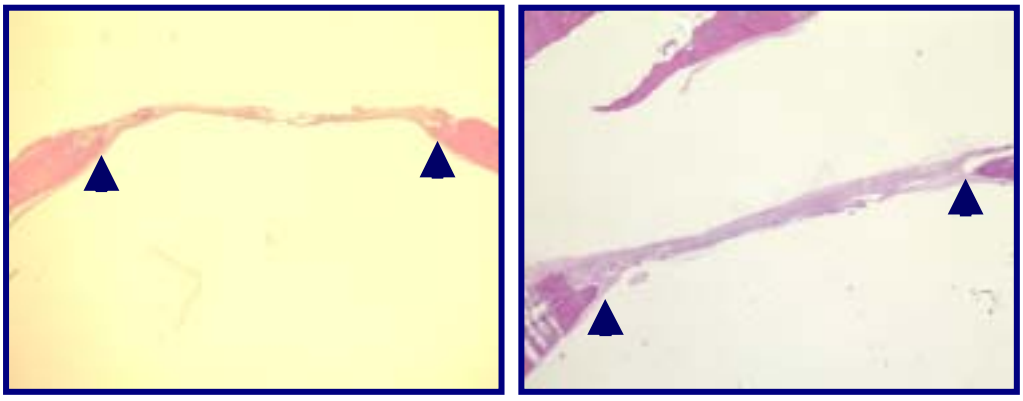

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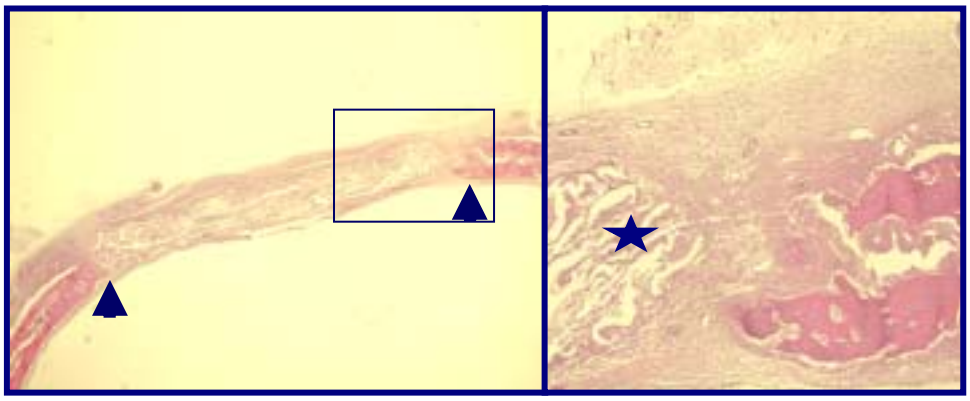
Figures (III)



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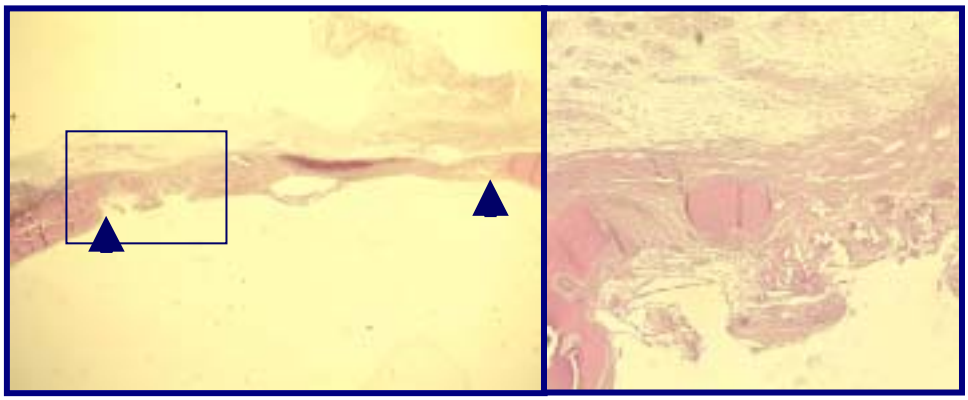

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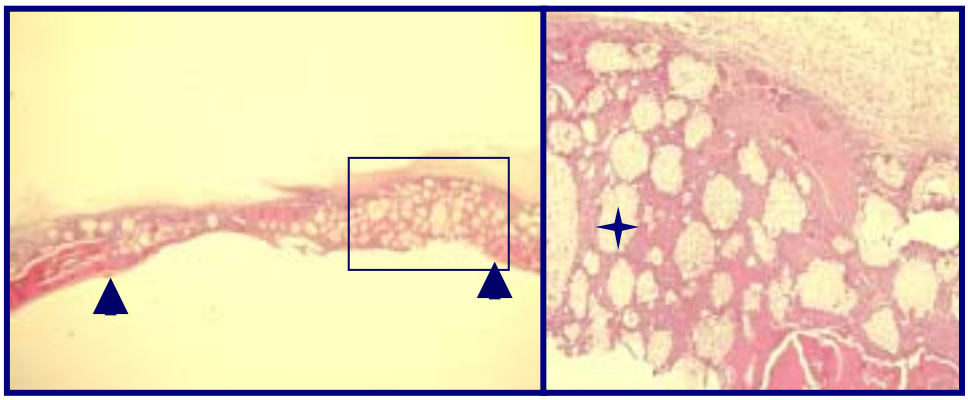
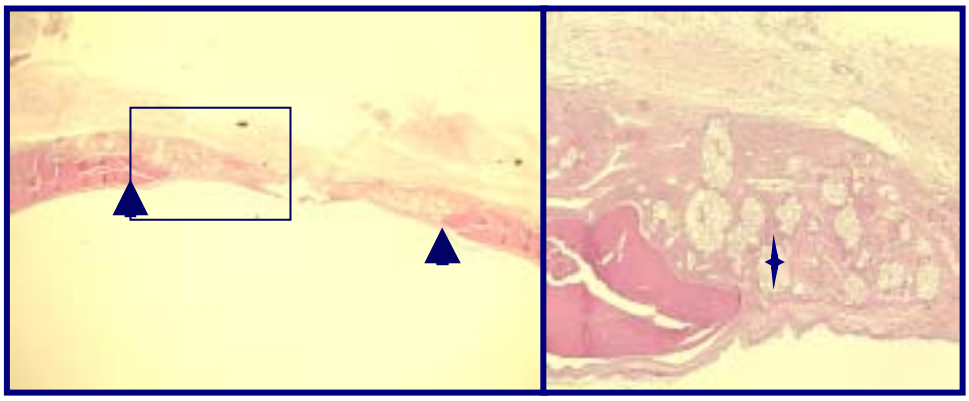


Figure 9.

Figures (IV)



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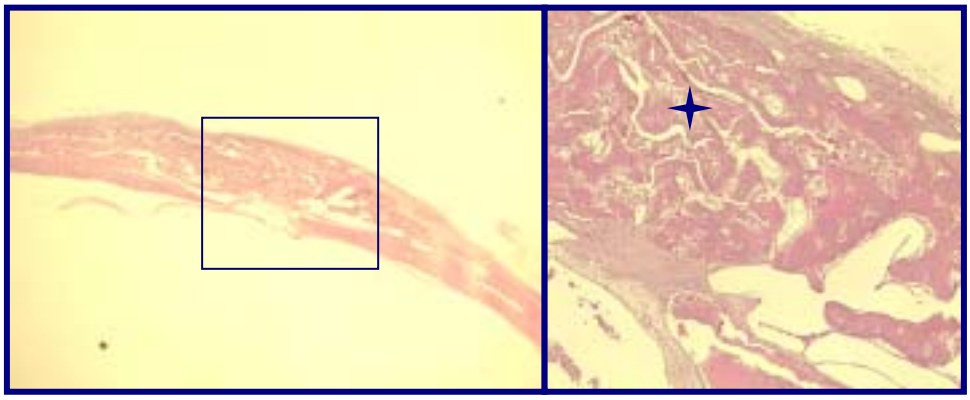
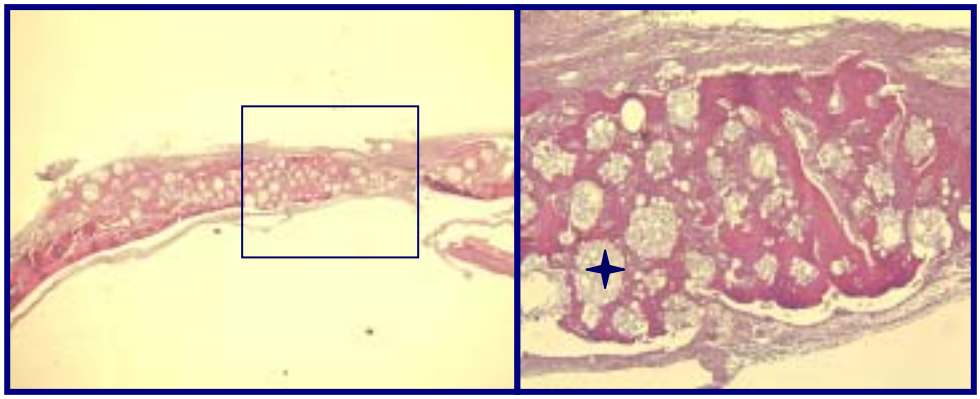


Figure 11.

Figure 12.

Figures (V)



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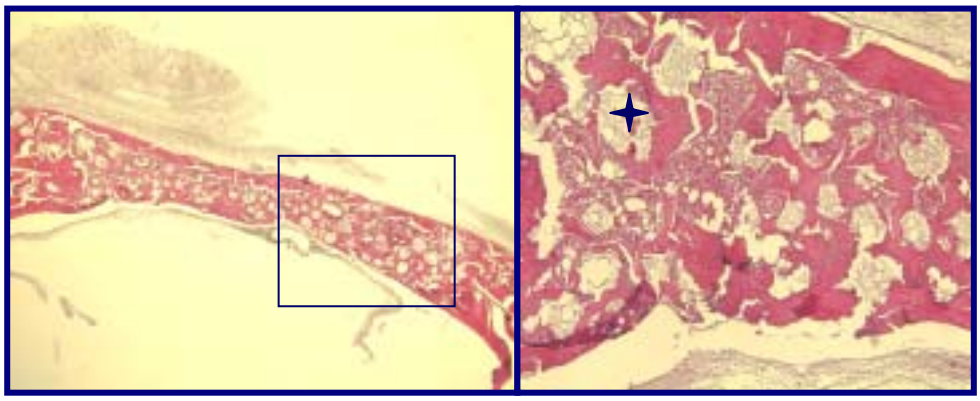

Figure 14.

국문 요약

백서 두개골 결손부에서 rhBMP-4와 운반체에 의한 골 재생 효과

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골형성 유도 단백질(bone morphogenetic protein, BMP)은 치주 치료와 골 재생 치료를 위한 골 대체 물질의 하나로 연구, 평가되어 지고 있다. rhBMP에 대한 연구 중 rhBMP-4의 골 형성 유도 효과와 운반체에 대한 연구가 아직 부족한 편이다. 이에 본 연구에서는 흡수성 콜라겐 스폰지(ACS)와 베타 삼화인산칼슘염(β-TCP)을 운반체로 하여 백서 두개골 결손부에서의 rhBMP-4의 골형성 유도 효과를 평가하고, rhBMP-4의 운반체로서 ACS와 β-TCP를 비교하고자 한다. 본 연구에서 100마리의 웅성 백서를 5개의 그룹으로 나누고, 8 mm 백서 두개골 결손부에 rhBMP-4/ACS, rhBMP-4/β-TCP, ACS, β-TCP를 이식하고, 수술 대조군에는 아무것도 이식하지 않았다. 술후 2, 8 주에 실험 동물을 희생하고, 치유 결과를 방사선밀도계측학적으로, 조직학적으로, 조직계측학적으로 비교 관찰하였다.

방사선밀도계측학적 결과로는, 술후 2주에 rhBMP-4/ACS, rhBMP-4/β-TCP, ACS 대조군, β-TCP 대조군, 그리고 수술 대조군의 신생골 밀도(%) 계측 결과는 각각 55.0±7.2%, 56.7±7.3%, 37.9±7.0%, 39.5±5.1%, 15.3±2.9%와 같이 나타났으며, 술후 8주에는 각각 61.4±12.6%, 76.1±5.8%, 34.0±3.4%, 42.7±7.9%, 17.2±3.7%와 같이 나타났다. 2주와 8주에서 rhBMP-4/ACS와 rhBMP-4/β-TCP 는 다른 실험군에 비해 신생골 밀도가 더 높게 나타났다($P<0.01$). 8주에서 rhBMP-4/β-TCP는 rhBMP-4/ACS보다 더 높은 신생골 밀도를 보였다($P<0.01$).

조직학적 관찰 결과, rhBMP-4/ACS 군에서는, 술후 2주에 신생골 내에 ACS 분절이 묻혀 있는 양상을 보였으며, 술후 8주에 결손부가 신생골로 가득 메워졌으며, ACS는 관찰되지 않았다. rhBMP-4/β-TCP 군에서는, 술후 2주에 다수의 잔존 β-TCP와 신생골이 관찰되었으며 술후 8주에는 다소 흡수된 양상의 잔존 β-TCP와 신생골이 관찰되었다.

조직계측학적 결과로는, 술후 2주에 rhBMP-4/ACS, rhBMP-4/β-TCP, ACS 대조군, β-TCP 대조군, 그리고 수술 대조군의 신생골/결손부(%) 계측 결과는 각각 71.7±2.8%, 60.8±.2.0%, 13.6±2.3%, 15.3±.2.0%, 4.8±0.1%와 같이 나타났으며, 술후 8주에는 각각 91.9±2.6%, 75.9±2.3%, 17.4±2.5%, 20.2±3.0%, 8.2±0.0%와 같이 나타났다. 2주와 8주에서 rhBMP-4/ACS와 rhBMP-4/β-TCP는 다른 실험군에 비해 신생골/결손부(%)가 더 높게 나타났으며($P<0.01$), rhBMP-4/ACS는 rhBMP-4/β-TCP보다 더 높은 신생골/결손부(%)를 보였다($P<0.05$).

이상의 결과에서 볼 때, 백서 두개골 결손부에서 rhBMP-4/ACS, rhBMP-4/β-TCP를 적용한 경우, 신생골/결손부(%)와 신생골 밀도에 있어서, rhBMP-4의 골 형성 유도 능력을 확인할 수 있었으며, 또한 ACS와 β-TCP는 rhBMP-4의 운반체로서 효과적이라 사료된다.

핵심되는말 : 신생골 형성, 골형성 유도 단백질, 흡수성 콜라겐 스폰지(ACS), 베타 삼화인산칼슘염(β-TCP), 백서 두개골 결손부