

The Effect of Macroporous Biphasic
Calcium Phosphate Block as Carrier of
Recombinant Human Bone
Morphogenetic Protein-2 on Bone
Formation in Rat Calvarial Defects.

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This certifies that the dissertation thesis
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감사의 글

먼저 이 논문이 나올 수 있게끔 부족한 저를 지도해주시고 이끌어 주셨던 존경하는 조규성 교수님의 큰 가르침에 깊은 감사를 드립니다. 그리고 많은 관심과 격려를 해주신 김종관 교수님, 채중규 교수님, 최성호 교수님, 김창성 교수님에게도 진심으로 감사 드립니다.

연구 내내 많은 도움을 준 조익현 선생님과 한동관 선생님과 그리고 치주과 교실원 여러분께도 고마움을 전합니다.

또 항상 저에게 인생의 비전을 제시해 주시는 신흥의 이용익 사장님께도 깊은 감사의 말씀을 전합니다.

깊은 사랑과 염려로 저를 키워주시고 보살피 주시는 아버지, 어머니, 또 부족한 남편에게 큰 사랑과 아낌없는 도움으로 큰 의지가 되어주는 사랑하는 나의 아내와 나에게 항상 빛이 되어 주고 따뜻함이 되어주는 사랑하는 나의 두 딸 다인, 다운에게 진정으로 사랑과 고마움을 전합니다.

다시 한번 모든 분들께 고개 숙여 감사의 말씀을 올립니다.

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Abstract

The effect of macroporous biphasic calcium phosphate Blocks as carrier of recombinant human bone morphogenetic protein-2 on bone formation in Rat Calvarial Defects

Bone morphogenetic proteins (BMPs) are currently being evaluated as potential candidates for periodontal and bone regenerative therapy. In spite of the good prospect of BMP applications, an ideal carrier system for BMPs has not been identified. The macroporous biphasic calcium phosphate (MBCP) block can be used as a bone augmentation material because of its hardness and ability to generate space. The purpose of this study is to evaluate the effect of the MBCP block as an rhBMP-2 carrier in the rat calvarial defect model.

Eight-mm critical-size calvarial defects were created in 40 male Sprague-Dawley rats. The animals were divided into 2 groups of 20 animals each. The defects were treated with MBCP blocks alone or rhBMP-2/ MBCP blocks. Defects were evaluated by histological and histometric parameters following a 2- or 8-week healing interval (10 animals/group/healing intervals).

The new bone area of the MBCP/rhBMP-2 group was significantly greater than the MBCP control group at both 2 and 8 weeks ($p < 0.01$). The new bone area of the 8 week group was greater than the 2 week group in all treatment conditions.

The total augmented area did not change when all groups were compared.

In conclusion, the bone regenerative effect of MBCP/ rhBMP-2 was superior to MBCP blocks alone in the rat calvarial critical-sized defect model. Surgical implantation of rhBMP-2/ MBCP blocks may be able to regenerate bone in the rat calvarial critical sized defects without any side effects. In addition, MBCP blocks may be considered effective carriers of rhBMP-2.

Key Words : rhBMP-2, critical sized defect, macroporous biphasic calcium phosphate block, augmentation, carrier.

The Effect of Macroporous Biphasic Calcium Phosphate Block as Carrier of Recombinant Human Bone Morphogenetic Protein-2 on Bone Formation in Rat Calvarial Defects.

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I . Introduction

Bone morphogenetic protein-2 (BMP-2) is a member of the transforming growth factor- β superfamily of multifunctional cytokines. It induces bone formation^{5,24,16} and plays an important role in development³².

Implantation of BMPs alone does not induce bone formation because the protein rapidly diffuses from the site of implantation. Use of an appropriate carrier material is essential for the delivery, retention, and release of BMPs at defect site^{15,21,22,3}.

A carrier material should have the following qualities: biocompatible in order to minimize local tissue response, easy to mold to the desired shape, biodegradable for

its replacement by newly formed bone, enable the sustained release of BMP, and have mechanical stability in bone defects^{29,3,30,28,12,14,20,23}.

Many materials, such as tricalcium phosphate^{29,30,9,33} poly(lactic acid) polymer¹¹, absorbable collagen sponge (ACS)^{1,8,31}, demineralized bone matrix¹¹, hydroxyapatite (HA)^{17,21}, fibrin sealant¹⁰, poly(lactic-co-glycolic) polymer^{4,19}, and composites of these materials²³ have been used and evaluated as BMP carriers for the healing of bone defects.

Until now, rhBMP-2/ACS has been effective when used as an inlay, but it has the great limitation of onlay indication^{6,26,27}. Many studies have shown that the rhBMP carrier maintains augmented space firmly and has good bone regeneration.

In our previous study, we tried to find the proper rhBMP dose²⁵ that could be compared to rhBMP-2,4,7.¹³ We also continued to search for excellent carriers of rhBMP such as ACS, β -TCP, the fibro-fibronectin sealing system¹⁰, and their combination. However, no rhBMP carriers were hard enough to endure the compressive force.

Apparently, with the appropriate space-providing carriers or adjunctive devices, rhBMP-2 may not only induce clinically relevant bone formation for alveolar ridge augmentation, but has also been shown to have compressive forces in the craniofacial complex⁷.

In recent years, studies by Daculsi et al, have demonstrated the stability and effectiveness of the mixture of hydroxyapatite (HA) and beta-tricalcium phosphate (β -TCP). HA provides a good scaffold for the new bone to grow, but has poor regeneration potential. β -TCP has good bone regeneration potential, but is not able to provide sufficient space for bone growth.

Mixing HA and TCP permits the association between the physico-chemical properties of each compound. This process allows the manufacturing of materials with controllable resorption and bone substitution depending on the proportion of HA and β -TCP.

According to studies conducted by Lynch and Nery, Legeros et al, and Daculsi et al, the mixture of 60% HA and 40% β -TCP constitutes the ideal mixture for using macroporous biphasic calcium phosphate (MBCP) as a bone substitute. MBCPTM has a porous form required for the biological exchanges that are particularly essential for bone in growth and mineralization. A MBCP block has a 70% global porosity. The MBCP macropore structure will help ionic change, BMP diffusion, and the movement and retention of osteogenic cells. In addition, HA provides sufficient firmness to the MBCP block and maintains the bone augmented space. Furthermore, the MBCP block can be molded into any desirable shape. Therefore, the MBCP block can be used as bone augmentation material due to its hardness and space-generating ability.

The purpose of this study is to evaluate the effectiveness of the MBCP block as an rhBMP-2 carrier.

II. MATERIALS & METHODS

1. Animals

A total of 40 male Sprague-Dawley rats (weight 200-300 g) were used. 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.

Table-1 Experimental design

Group	week	Number of rat
MBCP block only	2 weeks	10 rats
	8 weeks	10 rats
rhBMP-2/ MBCP block	2 weeks	10 rats
	8 weeks	10 rats

2. rhBMP-2 implant construction

rhBMP-2[¶] was diluted to a concentration of 0.025mg/ml. For the rhBMP-2/MBCP block implant, a sterile MBCP block^Ω was loaded with 0.5ml of the rhBMP-2 solution (100 micron litter per 1 block). Following a 5-minute binding time, the implant was prepared to fit the defect.

3. Surgical procedure

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

[¶] R&D Systems Inc., Minneapolis, MN, U.S.A

^Ω Biomatlante Inc, France

^θ Ketalar®, Yuhan Co., Seoul, Korea

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

* 3i, Palm Beach Gardens, FL, USA

manner as described by Schmitz and Hollinger. Then, each animal received one of two experimental conditions: theMBCP block alone or the rhBMP-2/ MBCP block. The skin was sutured for primary closure with 4-0 coated Vicryl sutures^o

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

4. Histological and histometrical procedures

The animals were sacrificed by CO₂ asphyxiation at 2 and 8 weeks post surgery. Block sections including the surgical sites were removed. The Samples were placed immediately into vials and 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 augmented area 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^f coupled with a video camera on a light microscope^Σ. Sections were examined at 20x magnification. And then Three parameters were measured (figure-1).

- 1) Augmented area (mm²) was measured including new bone, the residual biomaterials, mineralized bone, fatty marrow and fibrovascular tissue.
- 2) New bone area (mm²) was determined by the newly formed bone area within the total augmented area.
- 3) Bone density was calculated as follows: Bone density (%) =
$$\frac{\text{New bone area}}{\text{Augmented area}} \times 100$$

^f Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA

^Σ Olympus BX50, Olympus Optical Co., Tokyo, Japan

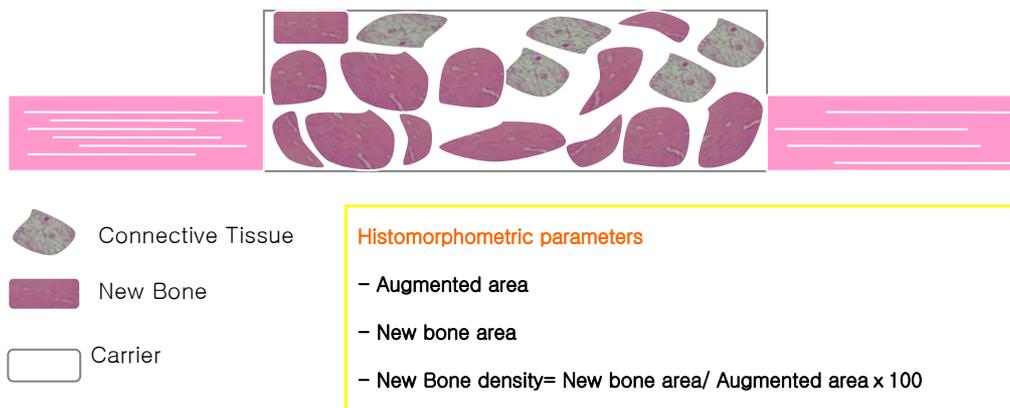


Figure 1. Schematic drawing of calvarial osteotomy defect showing the histometric analysis

5. Statistical Analysis

Histomorphometric recordings from the samples were used to calculate group means and standard deviation values ($m \pm SD$). To compare the 2 and 8week values in the same rhBMP, statistical significance was determined by a paired t-test. A two-way analysis of variance was used to analyze the effect of time and experimental conditions. The post hoc Scheffe's test was used to analyze the difference between the groups ($P < 0.01$).

III. Results

1. Clinical observation

Wound healing was generally uneventful, Two exposed MBCP block were observed which were excluded from the analysis.

2. Histologic observation

1) MBCP block group

At 2 and 8 weeks post surgery, the augmented areas were covered with dense, fibrous connective tissue. A small amount of new bone formation was observed adjacent to the margins of the defect at 2 weeks (Figure2). In addition, at 2 weeks a large number of residual MBCP particles were observed within the new bone.

However, at 8 weeks, in the downside of augmented area, many MBCP particles were embedded in or surrounded by newly formed bone and it was possible to observe the close contact between graft particles and the newly formed bone trabecules (Figure3).

Newly formed bone was characterized by lacunae containing osteoblasts, which appeared to be osteocytes, and had abundant medullary space filled with a well-vascularized connective tissue with no histological markers of inflammation or foreign body reactions.

2) MBCP/ BMP group

At 2 and 8 weeks post surgery, the augmented areas were more filled with new bone in the MBCP/BMP group than the control group. At 2 weeks, many osteoblasts and osteocytes were observed in the bottom of the MBCP block.

The quantity of new bone in the 8 week group was more than the quantity in the 2 weeks group, and the appearance of the new bone was more lamellar at 8 weeks than at 2 weeks. Concentric rings of the Haversian system, Cement lines, and fatty marrow were all observed in the new bone area (Figure 6). Newly formed bone grew from the bottom to the top of the MBCP block, and moved from the outside to the inside of the pore (Figure 5).

In particular, a great deal of new bone was observed at 8 weeks in the upside of augmented area (Figure7). In addition, in some cases the cranial bone of defect margin appeared to be covering the augmented material.

3. Histometric analysis

Two exposed block were excluded from the analysis and another 6 samples were excluded from the analysis because of technical problems.

The total augmented area did not change when all group were compared to each other (Table-2).

The new bone area of the rhBMP-2/MBCP block group was significantly greater than the MBCP block group at both 2 and 8 weeks ($p < 0.01$). The new bone area of the 8 week group was greater than the 2 week group in all treatment condition (Table-3). A two-way ANOVA revealed that there was an interaction between the healing interval and treatment condition in the new bone area ($p < 0.01$).

Table-2. Total augmented area (group means \pm SD, mm²)

	Total augmented area
MBCP (2weeks)	19.859 \pm 3.8
MBCP (8weeks)	20.346 \pm 2.7
MBCP+rhBMP-2 (2weeks)	22.419 \pm 3.4
MBCP+rhBMP-2 (8weeks)	22.373 \pm 4.1
No significant difference when compared to all groups ($P < 0.01$)	

Table-3. Bone density (group means \pm SD, N=8, %)

	New bone area/ augmented area * 100
MBCP (2 weeks)	7.27% \pm 3.7
MBCP (8 weeks)	14.74% \pm 5.2 [§]
MBCP+rhBMP-2 (2 weeks)	12.82% \pm 5.0*
MBCP+rhBMP-2 (8 weeks)	23.64% \pm 8.4 ^{§*}

§ : Statistically significant difference when compared to 2 weeks ($P < 0.01$)

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

IV. Discussion

Currently clinicians need bone augmentation on the alveolar ridge, due to alveolar ridge loss resulting from early tooth loss, surgical and accidental trauma, or pathologic processes. To resolve such problems, osteoconductive biomaterials or block implantation, autogenous bone grafts, and guided bone regeneration (GBR) were developed. Until now, autogenous bone grafts were considered to be the best. Autogenous bone grafts have many advantages, such as great osteogenic properties, histocompatibility, and the elimination of disease transmission.

However harvesting enough autogenous bone from an extraoral donor site requires general anesthesia, donor site operation, and hospitalization. For this reason, many researchers have been interested in rh-BMP.

The object of this study is to evaluate the effects of MBCP blocks as rhBMP-2 carriers when they are used in bone augmentation. The critical-size rat calvarial defect used in this study compared to other experimental bone defects is a convenient model for evaluating bone regenerative effects of biomaterials. This model is relatively accessible, simple, and reproducible because spontaneous healing does not occur in the control specimens (Frame, 1980; Schmitz et al., 1986). In addition, after bone augmentation, this model has some compressive force, which is similar to intraoral conditions.

Recently, the use of β -TCP has developed as an osteoconductive bone substitute and a biodegradable delivery system for rhBMP^{1,2,9,18}. β -TCP is porous and able to entrap rhBMP within its micropores, thus allowing the intrinsically diffusible rhBMP to be retained and its action prolonged²⁹. The porous structure of β -TCP allows cells and newly forming tissues to migrate into it, and also provides sufficient firmness against soft tissue pressure. However there are some limitations to the use of β -TCP. β -TCP is quickly resorbed, therefore making the maintenance of space difficult. In addition, manipulation is not easily achieved in β -TCP without any assistance.

MBCP blocks (consisting of a 60% HA and 40% β -TCP mixture) were expected to function better than the β -TCP material because of the addition of HA. Porous by nature, MBCP blocks would entrap rhBMP-2 within its micropores and macropores. 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 augmented area increased from 2 weeks to 8 weeks and because much of the carrier materials were resorbed at 8 weeks. There were statistically significant differences between the rhBMP-2/ MBCP group and the MBCP group. These findings showed that rhBMP-2 induced new bone formation in the augmented area.

In our study, the total augmented area had no change in all groups, meaning that the MBCP block is better than other materials as rhBMP carrier.

In the rhBMP-2/MBCP block group at 8 weeks, we saw new bone in the upside of the MBCP block, thus establishing that rhBMP is associated with osteoinduction.

In conclusion, the bone regenerative effect of the rhBMP-2/MBCP block was superior to the MBCP block in the rat calvarial critical-sized defect model. These findings may offer additional clinical uses, including dental implants, alveolar ridge augmentation, and other conservative treatments.

Nevertheless, more research is necessary on the sustenance of HA particles without resorption, and their residual influence.

V. Conclusion

The purpose of this study was to evaluate the osteogenic effects of macroporous biphasic calcium phosphate (MBCP) as a carrier system for rhBMP-2 in the rat calvarial defect model.

The bone regenerative effect of the rhBMP-2/MBCP blocks was superior to the MBCP blocks alone. Surgical implantation of rhBMP-2/MBCP blocks may be able to regenerate bone in the rat calvarial critical-sized defects without any side effects.

In conclusion, MBCP blocks may be considered effective carriers of rhBMP.

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Legends

Figure 1. Schematic drawing of calvarial osteotomy defect showing the histometric analysis.

Figure 2. Representative photomicrographs of MBCP block carrier control at 2 weeks. At 2 weeks, the augmented area were covered with dense connective tissue and particles of residual MBCP. Minimal new bone formation was observed. (x20)

Figure 3. Representative photomicrographs of MBCP block carrier control at 8 weeks. At 8 weeks, more bone formation was observed in the base area comparing to 2 weeks (x20)

Figure 4. Representative photomicrographs of rhBMP-2/MBCP block group at 2 weeks (x20). At 2 weeks, the augmented area are filled with new bone more than control group. A lot of osteoblast, osteocyte were observed in the bottom of MBCP block.

Figure 5. Representative photomicrographs of rhBMP-2/MBCP block group at 8 weeks (x20). Concentric rings of the haversian system, cement lines and fatty marrow were observed in the new bone area. At 8weeks, in the upside of augmented area, a lot of new bone was observed, and the appearance of the new bone was more lamellar at 8 weeks.

Figure 6. Representative photomicrographs of rhBMP-2/MBCP block group at 8 weeks (base of MBCP block, x100).

Figure 7. Representative photomicrographs of rhBMP-2/MBCP block group at 8 weeks (Top of MBCP block, x100). A lot of new bone was observed in top of MBCP block.

Figures I

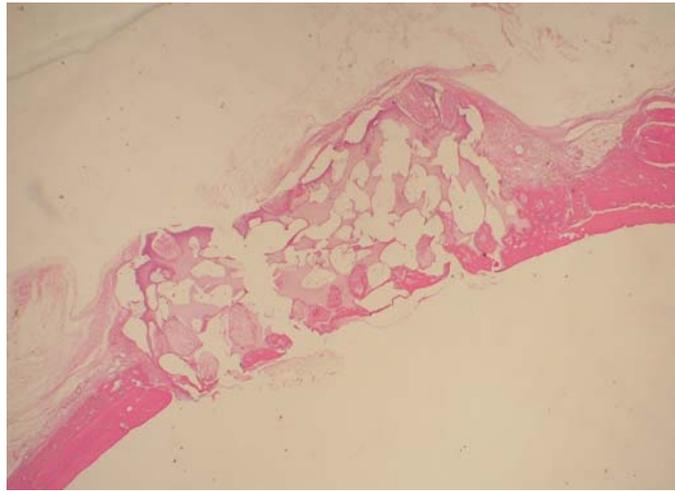


Figure 2

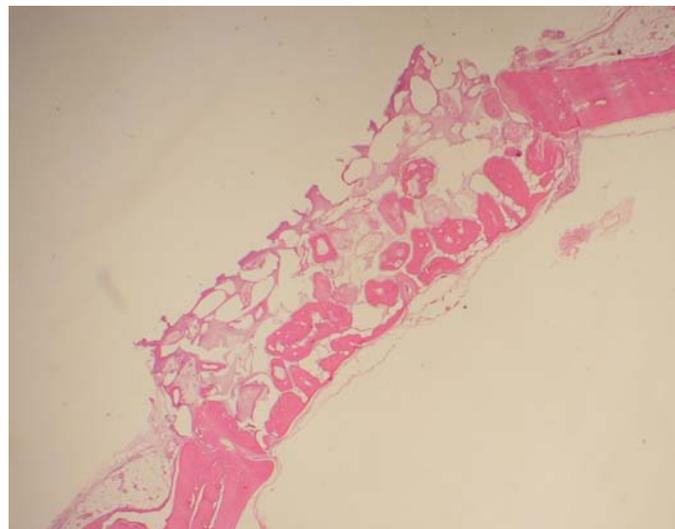


Figure 3

Figures II

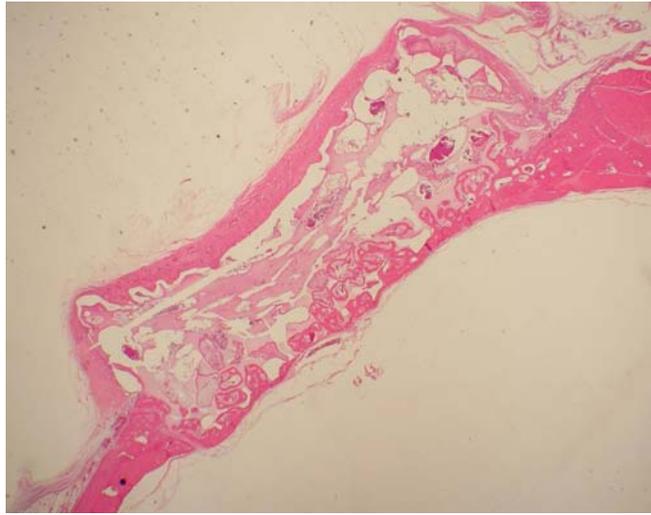


Figure 4

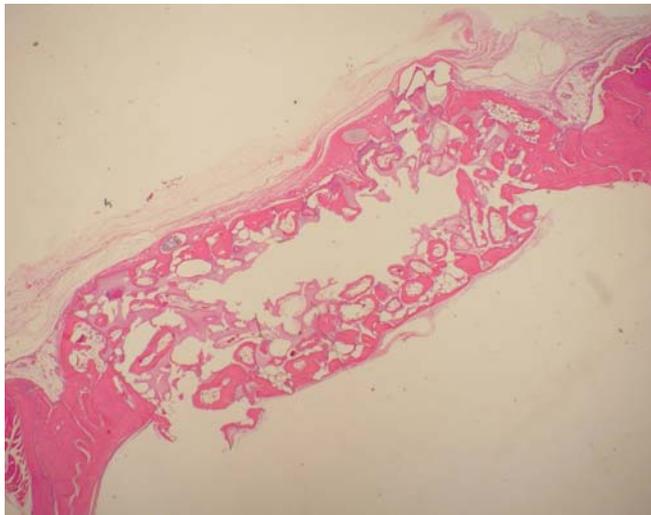


Figure 5

Figures III

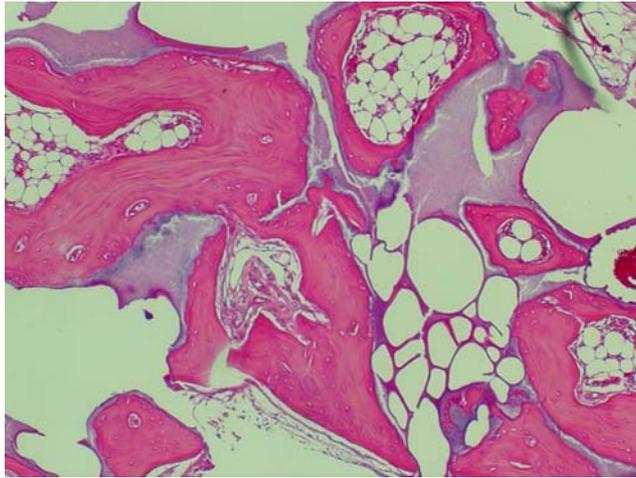


Figure 6

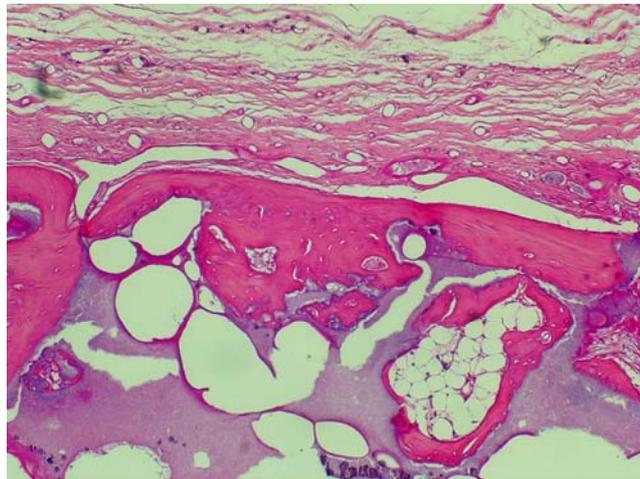


Figure 7

국문요약

백서 두개골 결손부에서 **bone morphogenetic protein-2**의 전달체로서 **macroporous biphasic calcium phosphate-block**의 골재생효과

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골형성 유도 단백질 (bone morphogenetic protein, BMP) 은 성장이나 골형성 과정에서 중요한 역할을 한다고 입증 되었고 그것의 운반체에 대한 연구가 이뤄져 왔다. 하지만 수직압이 존재하는 곳에서 골증대술에 적용할 수 있을 만큼 강한 공간유지능력이 있는 운반체에 대한 연구는 그리 많지 않았다. macroporous biphasic calcium phosphate Block (MBCP block)은 공간유지능력이 뛰어나며 강한 수직압을 견딜수 있는 골대체물질이다. 이 연구의 목적은 MBCP block을 골형성 유도 단백질 (rhBMP-2)의 운반체로 사용하여 백서 두개골 결손부에 적용하였을 때, 골형성 효과를 평가하는 것이다.

36 마리의 웅성 백서에서 8 mm 지름을 갖는 임계크기의 두개골 결손을 형성하였다. 20 마리씩 2 개의 군으로 나누어 MBCP block 만 이식한 군, MBCP block 을 운반체로 사용하여 농도 0.025mg/ml rhBMP-2 를 이식한 군으로 나누어 술 후 2 주와 8 주에 치유 결과를 조직학적, 조직계측학적으로 비교 관찰하였다.

조직계측학적 관찰 결과, rhBMP-2/MBCP block군에서 MBCP block군에서 보다 2,8주 모두 골밀도 (bone density)가 유의성 있게 증가하였다 ($P<0.01$). 각군에서도 8주째가 2주째보다 골밀도가 유의성있게 증가하였다 ($P<0.01$). 총조직 형성량 (augmented area) 에서는 변화가 없었다. 백서 두개골 결손부에서 MBCP block은 rhBMP의 운반체로 사용하였을 때 신생골 형성에 유의한 효과가 있을 뿐 아니라 공간유지능력이 우수해서 수직압이 존재하는 골증대술 (bone augmentation) 시 rhBMP의 운반체로 가능성이 있다.

핵심되는 말: 골형성 유도 단백질, macroporous biphasic calcium

phosphate Block, 운반체, 골증대술, 백서 두개골 결손부.