Bone formation using freeze-dried rhBMP-2 coated macroporous biphasic calcium phosphate blocks in rat calvarial defects

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Bone formation using freeze-dried rhBMP-2 coated macroporous biphasic calcium phosphate blocks in rat calvarial defects

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This certifies that the dissertation thesis
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June 2011

감사의 글

멀고 힘들게만 느껴졌던 긴 여정 끝에 이 논문의 완성을 앞두고 혼자서는 결코 이루지 못하였으리라는 생각에 많은 도와주신 분들에게 감사의 글을 드리고자 합니다. 먼저 많이 부족한 제자를 이끌어 주시고 격려해주신 조규성 지도교수님께 깊은 감사를 드립니다. 또한 논문을 심사해주신 김종관 교수님, 채중규 교수님, 최성호 교수님, 윤정호 교수님께도 고마움의 인사를 드립니다. 힘들었던 동물실험을 도와주시고 컴퓨터 작업등 세세한 도움을 주신 연세대학교 치주과 의국원 여러분께도 감사 드리며 특히 장지웅 선생님과 정임희 선생님께 고마운 마음을 전합니다. 보훈병원 치과진료센터에 박필규부장님과 과장님들, 치주과에서 같이 일하고 있는 유미경 선생님, 이동운 선생님, 수련의 선생님들께도 그동안 배려해주심에 대해 감사의 말을 전하고 싶습니다.

작년에 소천하신 친정 아버님과 힘든일 뒤에도 여전히 사랑을 느끼게 해주시는 친정 어머님과 동생들, 손주들 뒤바라지에 힘써주신 어머님과 누구보다 외조에 뒤지지 않는 사랑하는 남편과 너무 예쁜 다인, 세정이에게도 사랑과 고마움의 인사를 드립니다

2011 년 6 월

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Abstract

Bone formation using freeze-dried rhBMP-2 coated macroporous biphasic calcium phosphate blocks in rat calvarial defects

The purpose of this study was to evaluate bone formation using macroporous biphasic calcium phosphate block (MBCP block) coated via either freeze-drying or adsorption with recombinant human bone morphogenetic protein-2 (rhBMP-2).

Calvarial defects (8mm in diameter) were treated with the following experimental conditions: MBCP block (0.13 g) alone (control), MBCP block coated with rhBMP-2 via adsorption (0.1 mg/ml), and MBCP block coated with rhBMP-2 via a freeze-drying procedure (0.1 mg/ml). The healing period was either 2 or 8 weeks.

After a 2-week healing period, the new bone formation differed significantly between those treated with an MBCP block with a freeze-dried coating of rhBMP-2 and the control group. With further healing (i.e., 8 weeks), the distribution of newly formed bone increased in both the freeze-dried coating group and the adsorption group. The new bone area and bone density of all groups differed significantly between the 2- and 8- week healing period.

The findings of the present study suggest that freeze-drying is a reliable method of incorporating rhBMP-2 onto MBCP blocks, for use in stable bone formation.

Key words: bone morphogenetic protein, freeze-drying, coating, bone formation

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I. INTRODUCTION

Bone regeneration in reconstructive surgery for bone regeneration is used widely in the fields of orthopedics, cosmetic surgery, and dentistry to restore congenital or acquired damage to the bone structure. The materials used for bone regeneration are classified as osteogenetic, osteoconductive, and osteoinductive, depending on the reqired bone-forming ability. Of these, osteoinductive materials elicit bone formation by promoting development of undifferentiated mesenchymal cells into bone cells. Growth factors, such as bone morphogenetic protein (BMP), have been the focus of recent attention because of their bone inducing- properties. In the mid-1960s, Urist demonstrated that this extract from mineralized bone could induce new bone formation[1]. It has been demonstrated that recombinant human BMP (rhBMP),

synthesized from Chinese hamster ovarian (CHO) cells, can induce ectopic bone formation [2-3].

More than 20 rhBMPs have been identified. Since they are supplied mainly in the liquid state, rhBMPs are not only difficult to manipulate, but also rapidly absorbed into the implanted area. Therefore, they need a scaffold to allow their slow release and absorption into the body. We have shown previously that rhBMP-2 used with various scaffolds appears to be a candidate tool for osteoinduction[4-6]. Ceramic carriers, which are currently being magnified, enables vascular and cellular ingrowth and is desirable for the definition of the spatial configutration of the induced bone[7]. Research on this material has progressed in the areas of oral reconstruction and orthopedics [8-11].

Because ceramics often do not possess bioactive properties, there has been increasing interest in the immobilization of BMP onto these materials, thus transforming them into biomimetic scaffolds[12-16]. Luginbuehl et al. reported on the benefits of adsorption methods, which are their simplicity and high sensitivity to environmental conditions. BMPs need to be carried and released in a controlled and sustained way , rather than in a burst[17].

Burst release produces a transient, local, high dose of BMP, thus overstimulating the osteoclastic activity[18]. Furthermore, Liu et al. reported that release of adsorbed BMP occurs too rapidly to be effective in a physiologic environment[19].

Previous studies tested the adsorption of rhBMP-2 onto various carriers by soaking the carrier in an rhBMP-2 buffer solution[20-22]. We have recently investigated

the efficacy of bone formation in vivo after using a freeze-drying method for coating *Escherichia-coli*-derived rhBMP-2 onto a ceramic block[23]. The resulting good bone formation has prompted considerable interest in this freeze-drying method, and how it compares to ceramic blocks to which rhBMP-2 has been adsorbed. There have been few experiments on carriers with a freeze-dried coating of rhBMP-2.

The purpose of this study was thus to compare the new bone formation achieved in rat calvarial defects using macroporous biphasic calcium phosphate (MBCP) blocks coated with rhBMP-2 by freeze drying and by adsorption.

I. MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats (*n*=48; body weight 200-300 g) were used for the experiments. Animals were maintained in plastic cages in a room with a 12-h day/night cycle and an ambient temperature of 21°C, with *ad libitum* access to water and a diet of standard laboratory pellets. Animal selection and management, the surgical protocol, and preparation were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea. The 48 animals were divided into three groups (*n*=16 in each): one control and two experimental groups. The healing period was either 2 or 8 weeks (*n*=8 per group for each healing period). The control group was provided with an uncoated MBCP block, while the two experimental groups were given either an MBCP block coated with whBMP-2 by adsorption of an MBCP block coated with rhBMP-2 by a freeze-drying method.

2. Materials

2.1. Preparation of rhBMP-2 adsorbed MBCP blocks

Disc-shaped biphasic calcium phosphate (MBCP plus®; 3mm high and 8mm in diameter, weighing 0.13g) implants (Biomantlante, Nantes, France) comprising hydroxyapatite/beta-tricalcium phosphate at a ratio of 20/80 were manufactured. The

overall porosity was approximately 75%. CHO cell-expressed rhBMP-2 (Korea Bone Bank, Seoul, Korea) was reconstituted and diluted in a buffer solution to a concentration of 0.1 mg/ml. The block was loaded with 0.1 ml of the rhBMP-2 solution to obtain an implanted amount of 10 μg of rhBMP-2 into each defect. After allowing 60 minutes for the rhBMP-2 to adsorb to the surface of the MBCP block, it was implanted into a preformed calvarial defect.

2.2. Preparation of rhBMP-2 coated MBCP blocks

CHO cell-expressed rhBMP-2 solution (0.1 ml of 0.1 mg/ml buffer) was dispersed onto the MBCP block using a pipette. The block was then freeze-dried at –70℃ overnight, sterilized using ethylene oxide gas, and stored at 4℃ until animal implantation. The final amount of rhBMP-2 inserted into each defect was 10 μg.

3. Surgical protocol

Animals were anesthetized with an intramuscular injection (5 mg/kg body weight) of a 4:1 solution of ketamine hydrochloride (Ketalar®, Yuhan Co., Seoul, Korea). After shaving and scrubbing the surgical area, an incision was made in the sagittal plane across the cranium, and a full-thickness flap was reflected. A standardized, circular, transosseous defect, 8 mm in diameter, was created on the cranium with a trephine drill (3i Implant innovation, Palm Beach Gardens, FL, USA) and was copiously irrigated with saline. After removal of the circular disc, an MBCP block,

either uncoated (control) or coated with rhBMP-2 via either adsorption or the freeze-dried protocol (depending upon the experimental group), was loaded into the defect sites. All surgical sites were sutured for primary closure using 4-0 Monosyn® (glyconate absorbable monofilament, B-Braun Aesculap, PA, USA)

4. Histological and histometric analysis

Block samples were obtained at the 2- week (*n*=8 animals per group) and 8- week (*n*=8 animals per group) healing points. These samples were rinsed with sterile saline and fixed in 10% buffered formalin for 10 days. After rinsing with water, the samples were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Two 5-μm-thick serial sections were prepared from the center of each sample and stained with hematoxylin and eosin for histological evaluation. Specimens were examined under a binocular microscope (Leica DM LB, Leica Microsystems., Wetzlar, Germany) equipped with a camera (Leica DC300F, Leica Microsystems., Heerburgg, Switzerland). Images of the slides were acquired and saved.

After histologic analysis, computer-assisted histometric measurements were obtained using an automated image-analysis system (Image-pro Plus, Media Cybernetics, Silver Spring, MD, USA) in the calvarial defect model. Three parameters were measured: total augmented area, new bone area, and bone density. New bone area was measured as newly-formed mineralized bone, excluding marrow and fibrovascular tissue. Bone density was measured by calculating the percentage of

new bone area in the total augmented area, including all tissues within the boundaries of the defect (i.e., mineralized bone, fibrovascular tissue, bone marrow, and residual carrier biomaterial).

5. Microcomputed tomography

Block samples were fixed in 10% buffered formalin for 10 days and then scanned in a microcomputed tomography (micro-CT) system (Skyscan® 1072, Skyscan, Aartelaar, Belgium) at a resolution of 35 μm (100 kV and 100 μA). 3-D images were captured using OnDemand 3D® software (Cybermed, Seoul, Korea).

6. Stastistical analysis

Histomorphometric measurements of the samples were used to calculate the mean±SD values of each group. A one-way analysis of variance was used to test the effects of the experimental conditions. The post-hoc Scheffé test was used to analyze differences between groups, and an independent *t*-test was used to compare the difference between 2 and 8 weeks for each group. The level of statistical significance was set at $P<0.05$. All statistical calculations were performed using commercially available software (SPSS 15.0, SPSS ., Chicago, IL, USA).

III. RESULTS

1. Clinical observations

Healing was uneventful for all animals during the postoperative period. Two rats were excluded from the analysis due to technical deficiencies during the production process of the tissue specimens (rhBMP-2 adsorbing, one at 8 weeks; rhBMP-2 coating, one at 8 weeks).

2. Histologic evaluations

2.1. Uncoated MBCP block group

At 2 weeks of healing, there was little overall formation of new bone. The total augmented area was reduced compared to the rhBMP-2-adsorbed and rhBMP-2-freeze-dried groups. Connective tissues surrounding the upper areas of grafted bone could be observed, as well as occasional bone formation in the side and lower areas. At 2 weeks, the inner areas of the graft were either occupied by newly formed fibro-vascular tissue, or were empty (Fig. 3). At 8 weeks, the formation of numerous connective tissues and blood vessels could be observed, and the amount of new bone tissue had increased compared to at the 2 week time point.

2.2. rhBMP-2 -adsorbed/ -freeze-dried MBCP block groups

At 2 weeks after surgery, new bone tissues were observed growing from all boundaries of the graft. Compared to the control group, a stable external form of the block was observed and there was a clear increase in blood vessels and newly formed bone (Fig 3). Fig. 5 shows that high magnification microscopy revealed consecutive osteoblasts, which are responsible for bone growth, covering the osseous trabeculae. In addition, multinucleated giant cells were discovered at the inner surface of the bone defect. The bone defect margin communicated with the grafted block via newly formed osteoid tissue (Fig. 3d, Fig. 3f).

At 8 weeks postimplantation, intensely vascularized marrow cavities appeared throughout the defect. Appositional formation of new bone resulted in the deposition of intermediate cement lines (Fig. 4b).

3. Histometric evaluations

As indicated in Table 1, the new bone area and bone density at 8 weeks were significantly higher in the rhBMP-2-adsorbed/-freeze-dried MBCP groups than in the control group. At 2- weeks, these parameters differed significantly between the control and the rhBMP-2-freeze-dried MBCP groups, but not between the control and the rhBMP-2-adsorbed groups. Comparison of the findings at 2 and 8 weeks revealed significant differences in new bone area (mm^2) and bone density (%) between all of the groups.

4. Micro -CT evaluations

In the uncoated MBCP block (control) group, integration with the surrounding adjoining bone was incomplete at 2 weeks, but most of the defect was filled at 8 weeks (Fig. 2a, Fig. 2d). In the two rhBMP-2-coated block groups, compact radiopacity of the graft and natural integration of the grafted bone with the cranium was observed at 2 and 8 weeks after implantation (Fig. 2).

IV. DISCUSSION

Since rhBMP was first made from CHO cells by Wozney and his colleagues in 1988, several studies have demonstrated the biological activities of this growth factor. Many studies have demonstrated the superior osteoinductive capacity of rhBMP-2 [21, 24-25]. In a previous study, we found favorable bone formation following the application of rhBMP-2-coated MBCP block carriers compared to a control group with uncoated MBCP blocks[23]. The present experiments tested two methods of coating MBCP block carriers with rhBMP-2 in animals to determine if the coating method influences bone induction. We found that both rhBMP-2-adsorbed MBCP blocks and rhBMP-2- freeze-dried MBCP blocks induced good new bone formation. Specifically, the new bone area and bone density at 2 weeks postimplantation significantly greater for the latter than the control (uncoated block) group, while they did not differ significantly between the former. We can therefore assume that the freeze-drying coating method initially stabilizes bone formation in a way that the adsorption-coating method does not. One reason for this may be the stability of the incorporation of the rhBMP-2, which may prevent swift detachment from the block carrier. Liu et al reported that rhBMP-2 coating method induces persistent bone formation activity[19]. They used a biphasic calcium phosphate coating technique that induced the incorporation of rhBMP-2 by forming a crystalline layer on the biomaterial. In the present study, we made a coating of rhBMP-2 by forming

concentrated rhBMP-2 in the buffer solution, allowing it to flow onto the carrier, followed by overnight freeze-drying at -70℃ and subsequent storage at 4℃.

The retention of BMPs depends upon whether the protein is immobilized within the carrier or adsorbed onto the surface during its formulation[26]. BMPs are soluble, and if delivered in a buffer solution their clearance is rapid. Less than 5% of the BMP dose remains at the application site, whereas combinations of the proteins with gelatin foam or collagen showed increased retention ranging from 15% to 55% [24]. The adsorptive immobilization method of rhBMP-2 incorporation is generally chosen because of its ease of use. However, desorption can occur on the surface depending on environmental factors such as liquid phase composition, temperature, and pH[16].

"Burst release" of BMPs compromise early osteogenic activity[27]. Even though passive adsorption methods greatly benefit from their simplicity, conformational changes and denaturation are widespread, leading to loss of protein activity and irreversible binding of growth factors[26]. Further research using experimental animals regarding the influence of environmental and stress factors on the rapid release of BMPs is necessary. Although opinions regarding the bone formation associated with the level of BMP are not consistent, a common finding is that the amount of bone formation is influenced by the orthotopic or ectopic environment and the transplantation area.

While with the effects of BMP concentration do not vary in orthotopic environments such as the cranium, the amount of bone formation is proportionate to the concentration of BMP in the soft tissue environment[6, 28]. Jung et al. compared

rhBMP-2 amounts of 10 μg and 30 μg using a hydroxyapatitie (HA)-tricalcium phosphate (TCP) carrier and found no significant difference in bone formation between the two[7]. However, Schopper et al. showed that 10 μg of rhBMP-2 increased bone regeneration compared with the higher amount[29]. The present study found that, 10 μg rhBMP-2 was effective at increasing bone formation.

The HA-TCP carrier used in the present study is a type of ceramic material and related research has been actively progressing[29-31]. HA-TCP alone appears to have no osteoinductive ability, and so various methods of inducing bone formation have been tested using rhBMPs. We used a variable ratio of HA-TCP block carrier of 20:80. As the proportion of soluble TCP was relatively high, a fast absorption rate could be anticipated. Boden observed radiopaque material over a long period in samples, and early bone formation was found to be limited with high relative proportions of HA in carriers (e.g., 60:40), so they suggested that a relative proportion of TCP would lead to faster absorption of the biomaterial[32]. Likewise, we predicted fast absorption of the matrices because of the relatively high proportion of TCP compared to HA. As can be seen in fig. 3 and 4, there was marked new bone formation within the absorbed HA-TCP materials. Thus, the HA-TCP demonstrated stable bone inductivity as an rhBMP-2 carrier. This finding is similar to that of Alam, who found that an HA:TCP ratio of 25:75 induced more bone and marrow formation, and absorption of ceramic particles than did biphasic calcium phosphate ceramics with other component ratios.

It can also be seen in Fig. 4 that intensely vascularized marrow cavities were distinguishable in both of the rhBMP-2 experimental groups. We could therefore expect persistent bone formation even after only 8 weeks of healing.

The HA-TCP block carrier used in the present study has a characteristic form that is preferable to growth factor carriers: a 3-D structure and surface osteoinductivity, which support vascularization and bone ingrowth[17]. Allograft block carriers are superior to collagen with regard to their ability to maintain new bone formation[32-33]. Thus, it can be appreciated that 3-D structural integrity is an important factor. Fig. 3 and 4 show that the rhBMP-2-coated HA-TCP block was able to maintain the outer augmented area from 2 to 8 weeks after implantation, inducing good new bone formation. It can therefore be considered effective as an rhBMP-2 carrier.

V. CONCLUSION

After the early healing period (2 weeks), only the MBCP block with a freeze-dried coating of rhBMP-2 induced significantly greater new bone formation than did the control group. With further healing time (8 weeks), we observed good bone formation with both freeze-dried coating and the adsorption coating of the rhBMP-2 on MBCP blocks. We assumed that the reduced bone formation observed with the rhBMP-2-adsorbed MBCP in the early healing phase could be attributed to rapid release of the growth factor in this case, which could impair its osteogenic potency. We suggest that coating of an MBCP carrier with rhBMP-2 by freeze-drying is a reliable method of incorporating the growth factor to achieve stable bone formation.

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FIGURES

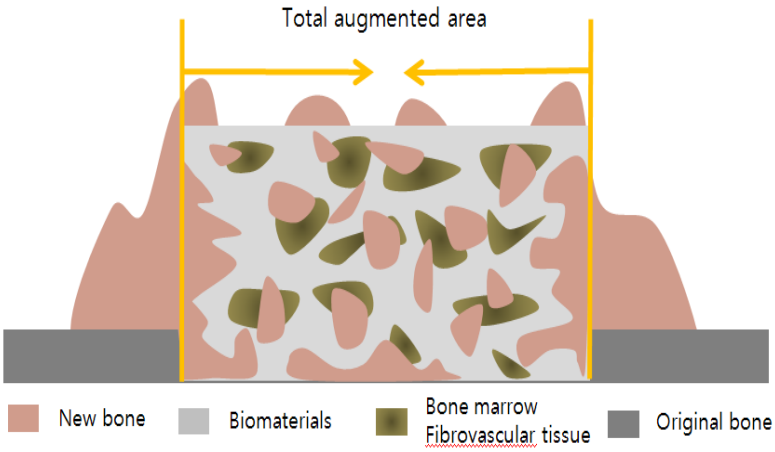

Figure 1. Schematic drawing of the histometric analysis of a calvarial defect model.

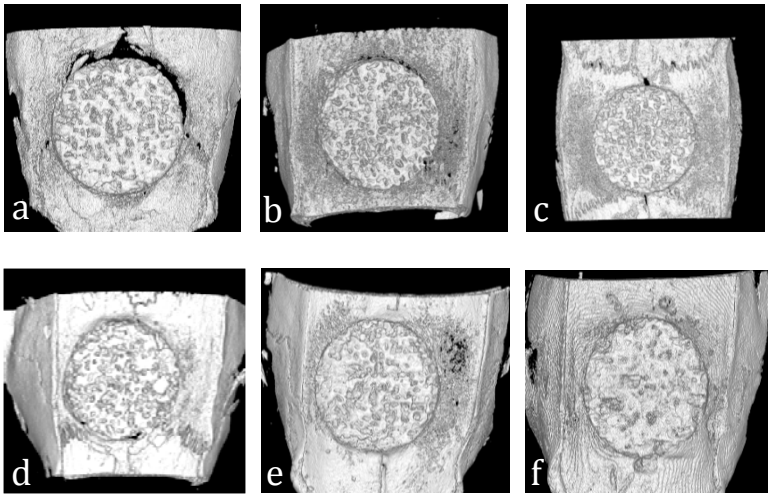

Figure 2. Representative micro-CT images of the defect sites at 2 and 8 weeks weeks postsurgery. At 2 weeks postsurgery: (a) MBCP block control; (b) 10-μg rhBMP-2- adsorbed MBCP block; (c) 10-μg rhBMP-2 freeze-dried coated MBCP block. At 8 weeks postsurgery: (d) MBCP block control; (e) 10-μg rhBMP-2-adsorbed MBCP block; (f) 10-μg rhBMP-2 freeze-dried coated MBCP block.

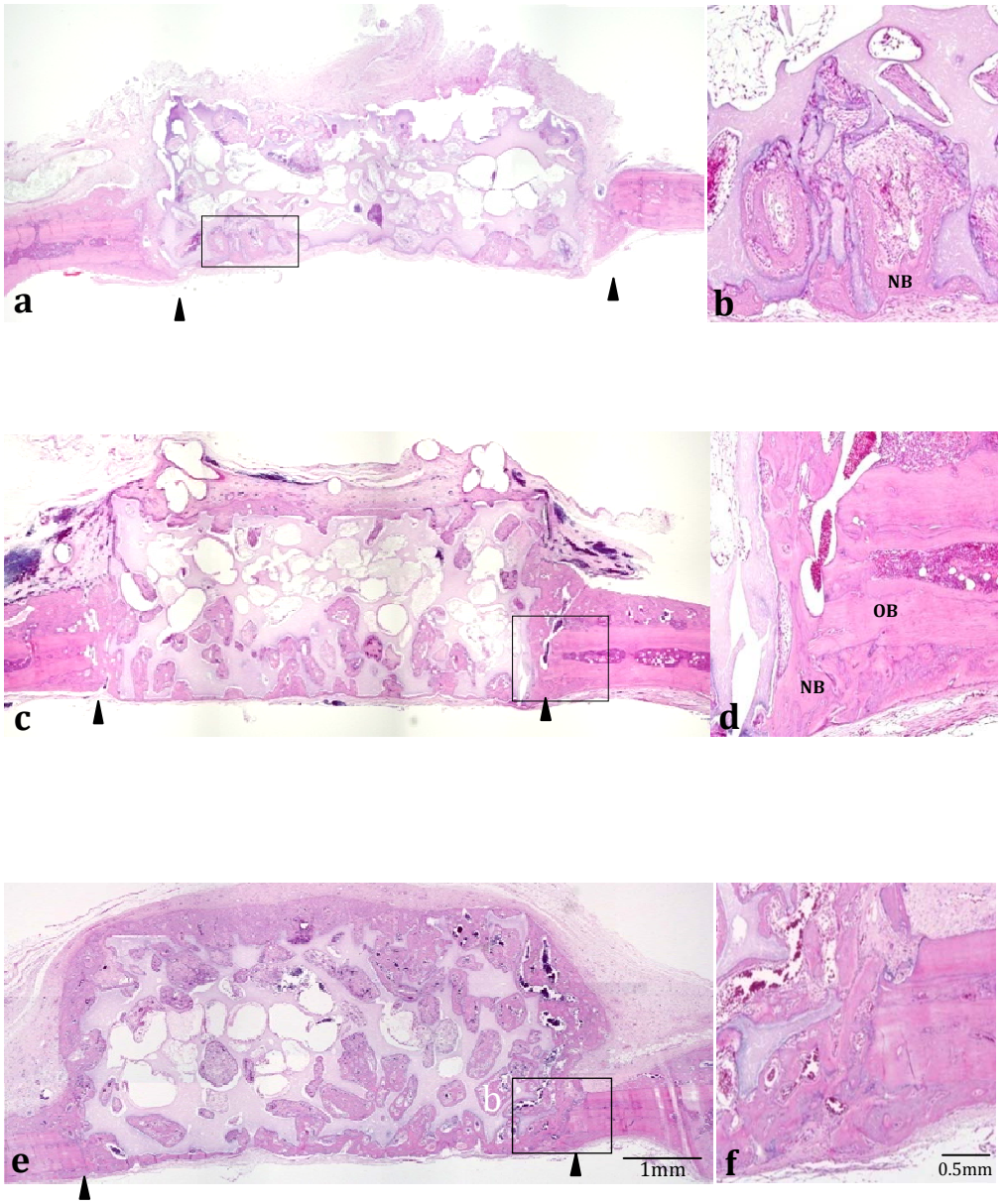

Figure 3. Representative photomicrographs of defect sites at 2 weeks postsurgery. (a,b) MBCP block control; (c,d) 10-μg rhBMP-2-adsorbed MBCP block; (e,f) 10-μg rhBMP-2 freeze-dried coated MBCP block. (arrow heads, defect margin; NB, new bone, OB, original bone; Hematoxylin and eosin (H&E) stain; original magnifications: a, c, and e ×40; b, d, and f ×100).

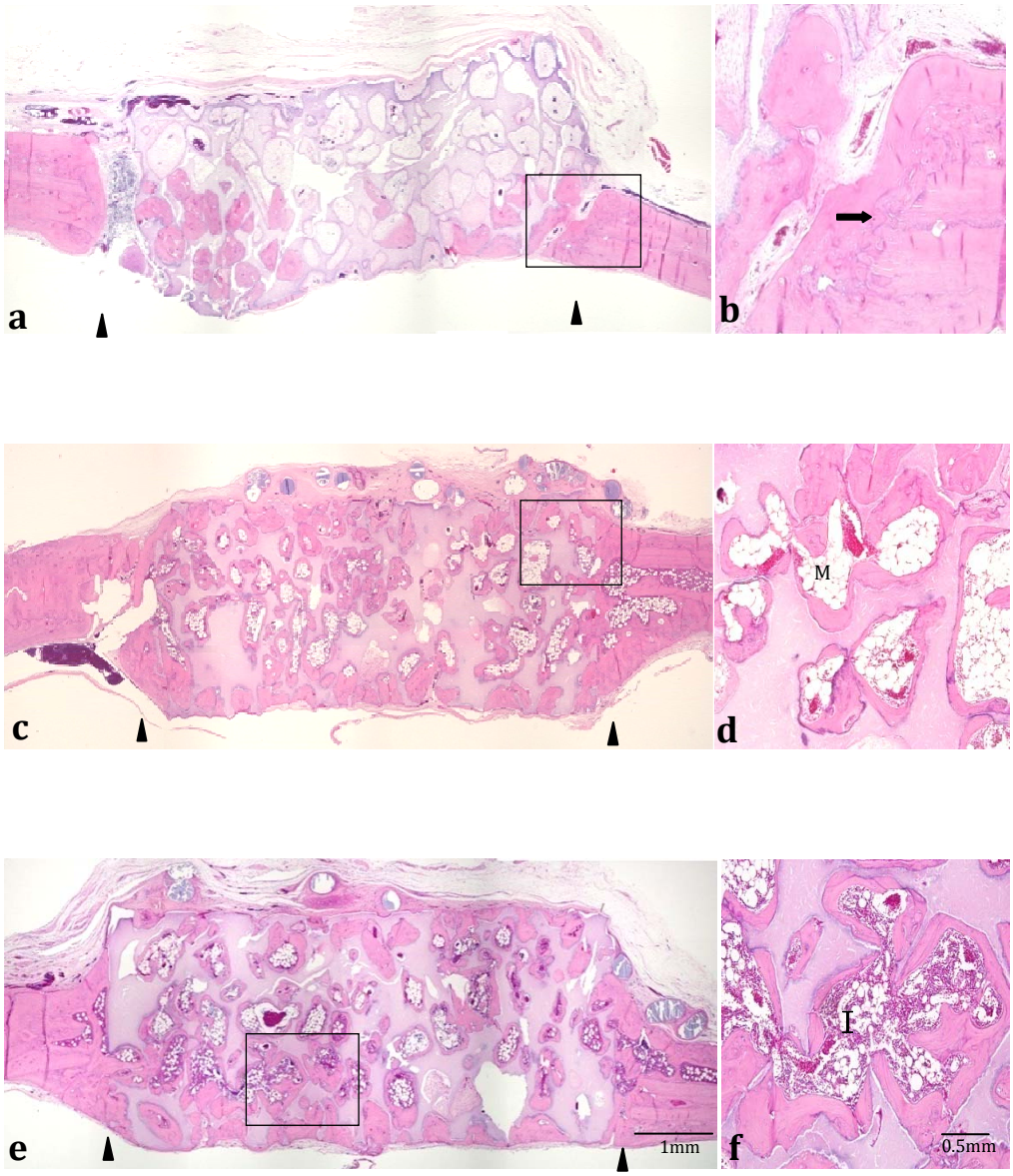

Figure 4. Representative photomicrographs of defect sites at 8 weeks postsurgery. (a) MBCP block control; (b) 10-μg rhBMP-2 adsorbed MBCP block; (c) 10-μg rhBMP-2 freeze-dried coated MBCP block. (arrow heads, defect margin; arrow, cement line; M, bone marrow; I, Intensely vascularized marrow cavity; H&E stain: original magnifications: a, c, and e ×40; b, d, and e ×100).

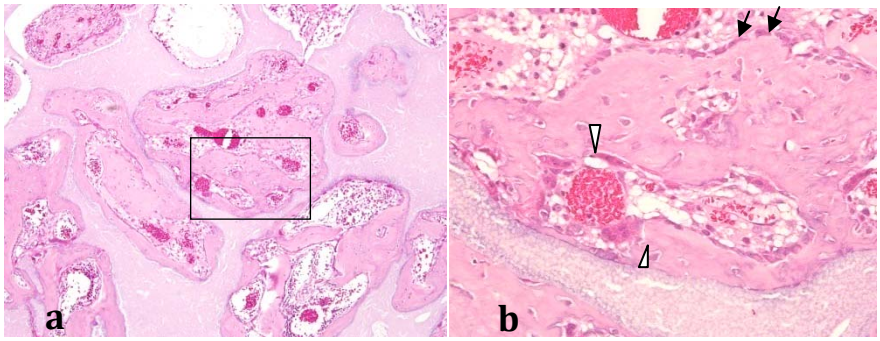

Figure 5. Representative photomicrographs of defect sites at 2 weeks. (a,b) 10-μg rhBMP-2 freeze-dried coated MBCP block. (white arrow head, multinucleated giant cell; arrow, osteoblast; H&E stain: original magnifications: a ×200; b ×400)

TABLE

Table 1. Histometric measurements at 2 and 8 weeks postsurgery

Group	Augmented area mean ± SD (mm^2)		New bone area mean ± SD (mm^2)		Bone density mean ± SD (%)	
	2 week (*n*=8)	8 week (*n*=8)	2 week(*n*=8)	8 week(*n*=7)	2 week(*n*=8)	8 week(*n*=7)
MBCP block control	15.42±2.68	17.71±2.56	0.16±0.23	1.15±0.87‡	1.04±1.46	6.62±4.75‡
RhBMP-2 -adsorbed	19.60±1.32¶	20.23±1.48	1.05±0.62	4.96±1.42¶‡	5.37±3.11	24.26±5.81¶‡
RhBMP-2 freeze-dried coated	20.04±1.56¶	20.11±1.71	1.76±1.38¶	4.47±1.26¶‡	8.75±6.88¶	22.25±5.69¶‡

*Bone density=new bone area/ augmented area × 100.

¶ :Stastically significant difference compared to the control group ($p<0.05$).

‡ :Stastically significant difference compared to 2 weeks postsurgery ($p<0.05$).

국문요약

백서의 두개부 결손부에서 macroporous biphasic calcium phosphate blocks 에 동결건조도포된 rhBMP-2 의 골형성능

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유 정 아

골재생을 위한 재건수술은 선천적, 후천적으로 손상된 골구조를 회복하기 위해 치과영역뿐만 아니라 정형외과 및 성형외과 영역에서도 광범위하게 진행되고 있다. 최근에는 다양한 성장인자 (growth factor) 들이 연구되어 적용됨으로써 골세포의 분화 및 골의 치유에까지 이르고 있다. 그 중, Recombinant human bone morphogenetic protein (rhBMP)의 재조합으로 BMP 의 사용영역이 넓어질수 있었다. rhBMP-2 의 액상 공급으로 인한 힘든 조작성과 빨리 흡수되는 특성으로 매개체를 필요로 하는데 본 교실에서는 adsorption 을 이용한 적용방법을 주로 이용하여 좋은 연구결과를 나타내었다. 최근 김등의 연구에서는 biphasic calcium phosphate blcok 에 coating 을 이용하여 rhBMP-2 를 적용한 후 많은 신생골 형성을 보였다. 이에 본 실험에서 adsorption 과 비교하여 백서의 두개골 부위에서 냉동건조도포법(freeze-dried coating method)을

이용하여 rhBMP-2 를 macroporous biphasic calcium phosphate block(MBCP block) carrier 에 적용한 후 나타나는 골유도능을 관찰해보고자 하였다.

총 48 마리의 백서를 3 군으로 나누어 두개골에 8 mm 의 골결손부를 형성하였다. MBCP block (0.13g) control, rhBMP-2-adsorbed MBCP block (0.1mg/ml), rhBMP-2 freeze-dried-coated MBCP block (0.1mg/ml) 군으로 나누어 술 후 2 주와 8 주 치유결과를 micro-CT, 조직학적 관찰과 조직계측학적 분석을 하였다.

연구 결과, 초기 치유 기간 후에는(2 주) 냉동건조도포군에서만 대조군에 비하여 유의한 차이를 나타내었고 충분한 치유 기간의 경과 후에는(8 주) 두 군 모두 훌륭한 골형성을 보였다. 2 주와 8 주의 기간에 따른 비교를 보면 대조군과 두실험군 모두에서 통계적 유의성을 나타내었다. 따라서 본 연구에서는 냉동건조 (freeze-dried)를 이용한 rhBMP-2 의 MBCP carrier 도포방법(coating)이 안정적인 골형성을 위한 신뢰성 있는 성장인자(growth factor)의 결합(incorporation)방법이라고 결론 지을 수 있었다.

핵심되는 말: 골유도단백질, 흡착, 냉동건조, 도포법, 골형성