

## rhBMP-2 using biphasic calcium phosphate block as a carrier induces new bone formation in a rat subcutaneous tissue

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### ABSTRACT

**Purpose:** The carrier for the delivery of bone morphogenetic proteins(BMPs) should also serve as a scaffold for new bone growth. In addition, predictable bone formation in terms of the volume and shape should be guaranteed. This study evaluated the ectopic bone formation of recombinant human BMP-2(rhBMP-2) using a micro macroporous biphasic calcium phosphate (MBCP: mixture of  $\beta$ TCP and HA) block as a carrier in a rat subcutaneous assay model.

**Materials and Methods:** Subcutaneous pockets were created on the back of 40 male Sprague-Dawley rats. In the pockets, rhBMP-2/MBCP and MBCP alone were implanted. The blocks were evaluated by histological and histometric parameters after a healing interval of 2 weeks (each 10 rats; MBCP and rhBMP-2/MBCP) or 8 weeks (each 10 rats; MBCP and rhBMP-2/MBCP).

**Results:** The shape and volume of the block was maintained stable over the healing period. No histological bone forming activity was observed in the MBCP alone sites after 2 weeks and there was minimal new bone formation at 8 weeks. In the rhBMP-2/MBCP sites, new bone formation was evident in the macropores of the block. The new bone area at 8 weeks was greater than at 2 weeks. There was a further increase in the quantity of new bone with the more advanced stage of remodeling.

**Conclusions:** A MBCP block could serve as a carrier system for predictable bone tissue engineering using rhBMPs.

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**KEY WORDS:** rhBMP-2; biphasic calcium phosphate block; carrier; ectopic bone formation.

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### Introduction

Since Urist<sup>1)</sup> demonstrated ectopic bone and cartilage formation after the intramuscular implantation of demineralized bone matrix in rats, which were later named as bone morphogenetic proteins(BMPs), several BMPs have been shown to have significant osteoinductive activity<sup>2-8)</sup>. It was reported that rhBMP itself is sufficient to induce bone formation. However,

the rapid diffusion of the water-soluble protein rhBMP from the implant site will reduce its osteoinductive effect. Therefore, a carrier system for rhBMPs is essential<sup>9-12)</sup>.

The carrier for BMPs should serve as a scaffold for bone forming cells while providing space in which bone formation can occur, and resist soft tissue compression during the healing period<sup>13)</sup>. For the clinically successful use of BMPs, the carrier should also be easy to manipulate, sterilize, and fabricate into the intended shape. It is also important that the shape and volume of newly formed bone be maintained. If these requirements of carrier are satisfied, predictable bone regeneration is possible with sufficient value to the tissue engineering side. There have been many

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studies on carriers<sup>14-17</sup>, but no carriers have satisfied these requirements perfectly.

Micro macroporous biphasic calcium phosphate (MBCP) consists of an intimate mixture of 40%  $\beta$ -tricalcium phosphate( $\beta$ -TCP) and 60% hydroxyapatite(HA)<sup>18,19</sup>. Besides the well-documented osteoconductive effect<sup>20-22</sup>, the osteoinductive effect of the MBCP is recommended for use as an alternative or as an additive to autogenous bone for orthopedics, traumatology, odontology and dental applications<sup>23-28</sup>. The porous structure, low resorption rate, favorable osteoconductivity and bioactive properties of MBCP can be utilized by rhBMPs carrier. The easy manipulation and producing the intended shape, particularly the block type, are additional merits of using this biomaterial. However, there has been little study on its use as a carrier for BMPs<sup>29,30</sup>.

The subcutaneous tissue of a rat has poorer conditions than the muscle or bony site or the subcutaneous tissue of other animals<sup>31-34</sup>. Therefore, success this region can indicate better success in other regions.

This study evaluated the ectopic bone formation of rhBMP-2 using a MBCP block as a carrier in a rat subcutaneous assay model.

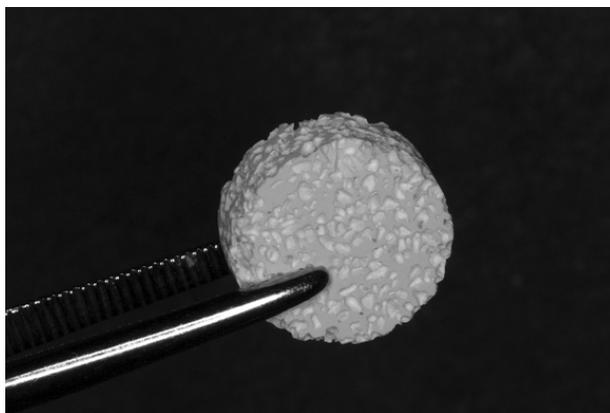
## Materials and Methods

### 1. Animals

40 male Sprague-Dawley rats (weight 250~300g, approximately 8 weeks old) were used. Rats were maintained in plastic cages in a room with a 12h-day/night cycle and an ambient temperature of 21°C, with *ad libitum* access to water and standard laboratory pellets. Animal selection and management, surgical protocol, and preparation were in accordance with the routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

### 2. rhBMP-2 Implant Construction

Disc-shaped MBCP implants (Biomatlante Inc., Vigneux de Bretagne, France) (3mm height and 8mm diameter) were manufactured (Fig. 1). rhBMP-2(R & D Systems Inc., Minneapolis, MN, USA) was reconstituted and diluted in a buffer to produce a concentration of 0.025mg/ml. For the rhBMP-2/MBCP implants, MBCP implants were loaded with 0.2ml of the rhBMP-2 solutions for one hour before surgery. For the MBCP implants alone, MBCP implants were loaded with 0.2ml of buffer solutions.



**Figure 1.** Block type MBCP implant used in this study (3mm in height and 8mm in diameter).

### 3. Surgical Procedures

The animals were anaesthetized by an intramuscular injection (5mg/kg body wt.) of a 4:1 solution of ketamine hydrochloride (Ketalar<sup>®</sup>, Yuhan Co., Seoul, Korea) and xylazine (Rompun<sup>®</sup>, Bayer Korea, Seoul, Korea). The surgical site was shaved and scrubbed with iodine. A vertical incision was made in the skin of the back. After flap reflection, a subcutaneous pocket was prepared by blunt instrument. Each animal received 1 of 2 experimental treatments: MBCP carrier control and rhBMP-2/MBCP. The skin was closed and sutured with absorbable monofilament suture (Monosyn<sup>®</sup>, Aesculap AG Co. KG, Tuttlingen, Germany).

### 4. Histologic and Histometric Procedures

The animals were sacrificed by CO<sub>2</sub> asphyxiation at 2 and 8 weeks post-surgery. Block sections were removed and fixed in a 10% neutral buffered formalin solution for 10 days. Samples were decalcified by 5% formic acid for 14 days and embedded in paraffin. Serial sections 7µm in thickness were prepared at intervals of 80µm, stained with hematoxylin/eosin (H-E), and examined using a light microscope. The most central sections from each block were selected for histologic and histometric evaluation.

Computer-assisted histometric measurements were obtained using an automated image analysis system (Image-Pro Plus<sup>®</sup>, Media Cybernetics, Silver Spring, MD, USA) coupled with a video camera on a light microscope (Olympus BX50, Olympus Optical Co., Tokyo, Japan). Sections were examined at magnifications of ×20 and ×100. Histometric parameters were defined as follows.

- Total augmented area (mm<sup>2</sup>): the area of implanted MBCP zone.
- New bone area (mm<sup>2</sup>): the area of newly formed bone within the total augmented area.

### 5. Statistical Analysis

Histometric recordings from the samples were used to calculate means and standard deviations ( $m \pm SD$ ). For comparisons between the two groups, a paired or unpaired t-test was used. The interactions between the healing times were examined using two-way analysis of variance. A p-value <0.01 was considered significant.

## Results

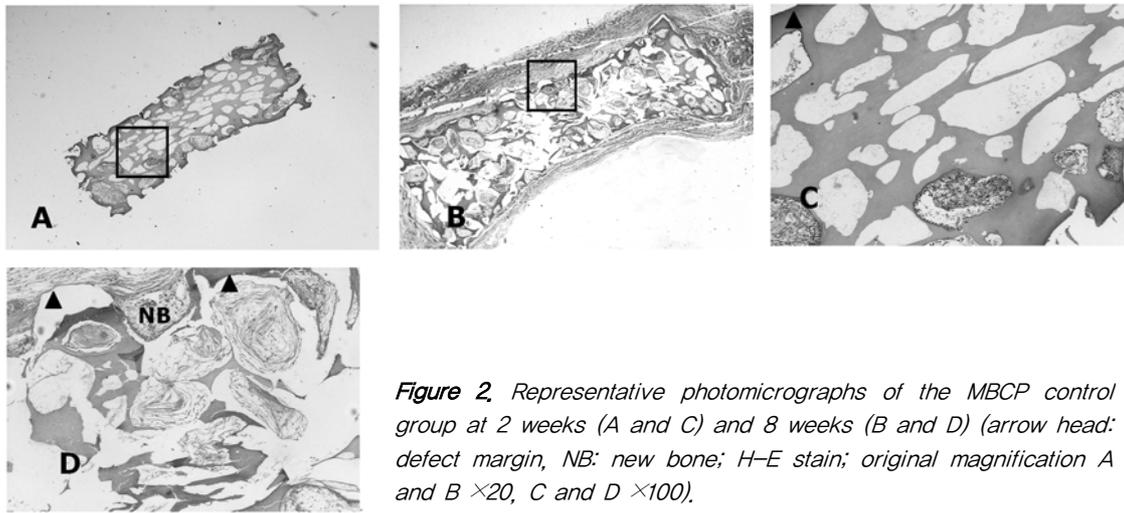
### 1. Clinical observation

Wound healing was generally uneventful and there were no signs and symptoms of infection or inflammation.

### 2. Histological Observations

#### 1) MBCP control group

At 2 weeks, histological signs of inflammation or foreign body reactions were not generally observed. MBCP blocks were surrounded by loose connective tissue. Macropores of the block were filled with connective tissue. Osteogenic activity could not be found histologically (Fig. 2-A, C). At 8 weeks post surgery, the implanted block was covered with dense and fibrous connective tissue. The minimal amount of new bone formation was observed adjacent to the margins of the block. No significant resorption of the MBCP block was observed during the healing time (Fig. 2-B, D).



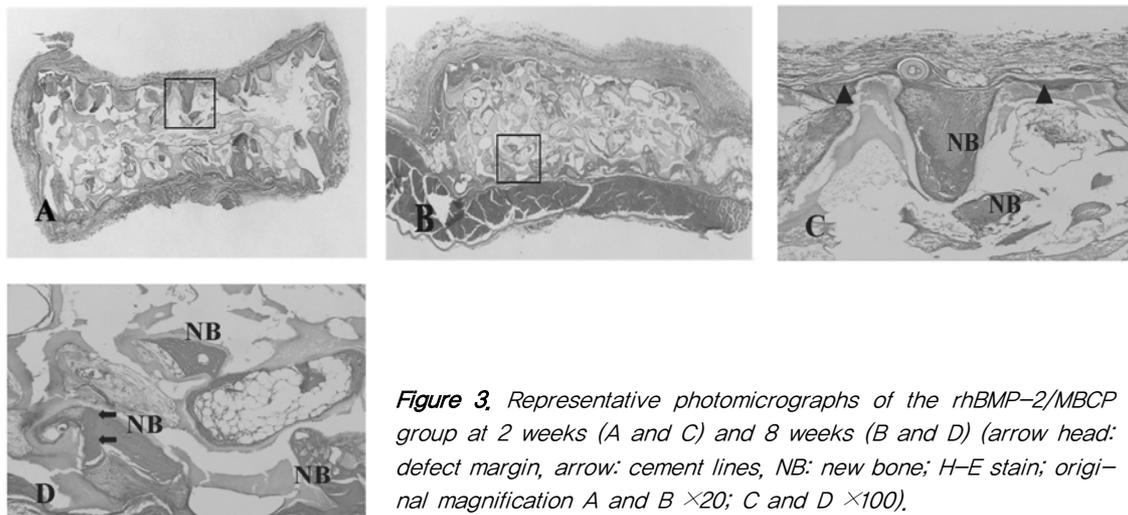
**Figure 2.** Representative photomicrographs of the MBCP control group at 2 weeks (A and C) and 8 weeks (B and D) (arrow head: defect margin, NB: new bone; H-E stain; original magnification A and B  $\times 20$ , C and D  $\times 100$ ).

2) MBCP/ rhBMP-2 group

At 2 weeks, the macropores in the periphery of the block were filled with new bone. Evidence of osteogenic activity, such as dense osteoblast-like cell lining, osteoid and bone apposition along the surface of macropores was observed. Macropores in the center part were usually filled with loose fibrous connective tissue and there was a little bone-forming activity (Fig. 3-A, C).

At 8 weeks, the quantity of new bone was greater

than that observed at 2 weeks, and the specimens showed a more advanced stage of remodeling and consolidation. Some macropores with fibrous connective tissue could be also found in the central part of the block. The newly formed bone consisted of woven and lamellar bone, and showed cement lines that were separated from the more recently deposited bone. There was no evidence of cartilage formation (Fig. 3-B, D).



**Figure 3.** Representative photomicrographs of the rhBMP-2/MBCP group at 2 weeks (A and C) and 8 weeks (B and D) (arrow head: defect margin, arrow: cement lines, NB: new bone; H-E stain; original magnification A and B  $\times 20$ ; C and D  $\times 100$ ).

**Table 1.** Total Augmented Area (group means ±SD, mm<sup>2</sup>, n=10)

Group	2 weeks	8 weeks
MBCP	21.9 ± 2.6	19.9 ± 2.8
rhBMP-2/MBCP	23.7 ± 1.9	23.5 ± 2.4

No significant difference when compared to all groups (P>0.01).

**Table 2.** New Bone Area (group means±SD, mm<sup>2</sup>, n=10)

Group	2 weeks	8 weeks
MBCP	0.8 ± 0.2	2.1 ± 1.1*
rhBMP-2/MBCP	3.9 ± 0.7¶	5.8 ± 2.4*¶

\*: Statistically significant difference compared to 2 weeks (p<0.01).  
 ¶: Statistically significant difference compared to MBCP group (p<0.01).

### 3. Histometric Analysis

Table 1 and 2 show the results of histometric analysis. In the rhBMP-2/MBCP group, the quantity of the new bone was greater at 8 weeks than 2 weeks (p<0.01).

### Discussion

The aim of this study was to evaluate the ectopic bone formation of rhBMP-2 using a MBCP block as a carrier in a rat subcutaneous assay model. Because the subcutaneous tissue of rats have poorer conditions than muscle or bony site or the subcutaneous tissue of other animals, the success in this region can indicate better success in other regions. The results were evaluated by histological and histometric analysis after a 2- and 8-week healing interval.

In a previous study<sup>13)</sup> using a similar method to this study, it was demonstrated that rhBMP-2, when impregnated in an absorbable collagen sponge(ACS) and β-TCP, provoked osteoinductive activity in rat subcutaneous tissue at 2 weeks. However, at 8 weeks neither ACS nor the newly formed bone observed at 2 weeks were found in the rhBMP-2/ACS sites, while more expanded bone maturation was observed in the

rhBMP-2/β-TCP sites. This suggests that ACS does not have capacity as a carrier for rhBMP-2 in this model. The lack of space-maintaining capacity of ACS might be one of the major factors responsible for the resorption of the newly-formed bone observed in the 2-week sections after implantation. It was concluded that the carrier for the delivery of BMPs should also serve as a scaffold for bone forming cells while providing sufficient space for bone formation to occur. Although favorable ectopic bone formation could be obtained using β-TCP particles, questions arise as to whether clinical applications are possible, because there is clinical difficulty in the nature of its particulate form, in the manipulation and maintenance of the intended shape of the bone. Since predictable bone tissue regeneration might be one of the major goals of the clinical therapeutic results, a carrier system for rhBMPs should provide stability over time in terms of volume and shape.

MBCP consists of an intimate mixture of 40% β-TCP and 60% HA, and is obtained when a synthetic calcium deficient apatite is sintered at ≥700°C. It is now available in blocks, particulates and as an injectable material in a polymer carrier<sup>18,19)</sup>. MBCP has excellent biocompatibility and bioactivity, which is related to its porous structure<sup>20-22)</sup>. Micropores ≤10μm allow fluid circulation, leading to the dissolution and

degradation of the biomaterial. Macropores  $\geq 100\mu\text{m}$  act as a scaffold for bone cells, thereby allowing centripetal bone ingrowth<sup>23)</sup>. The newly formed bone is in direct contact with the biomaterial surface, making this biomaterial osteoconductive. In addition to the well-documented osteoconductive effect, the osteoinductive effect of the MBCP is recommended for use as an alternative or additive to autogenous bone for orthopedics, traumatology, odontology and dental applications<sup>24,25)</sup>.

The porous structure, low resorption rate, favorable osteoconductive and bioactive properties of MBCP can be utilized by the rhBMPs carrier. The porous structure of MBCP can be retained, allow the slow release of rhBMPs, and provide the appropriate scaffold to allow cells and newly formed tissues to migrate into it. In clinical aspects, the MBCP block is a very interesting biomaterial for use as a carrier for BMPs because it can be manipulated easily to make the intended shape. However, there have been few studies on its use as a carrier for BMPs<sup>29,30)</sup>.

In this study, new bone formation in the macropores of the block was evident in the rhBMP-2/MBCP sites. The new bone area was greater at 8 weeks than at 2 weeks. The quantity of the new bone had increased further with the more advanced stage of remodeling. In addition, the total augmented area was stable during the healing period. In subcutaneous tissue of rats, this favorable results confirmed that the MBCP block is a good carrier system for tissue engineering using rhBMPs.

In conclusion, the use of the block type of MBCP as a carrier for rhBMP-2 is effective in inducing new bone formation. The new bone induced by the rhBMPs/MBCP block system was stable during the observed healing period. These results suggest that the MBCP block system can be used as a carrier system for tissue engineering using rhBMPs

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