Bone formation of block and particulated biphasic calcium phosphate lyophilized with *Escherichia coli*–derived recombinant human bone morphogenetic protein 2 in rat calvarial defects

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The objective of this study was to evaluate bone formation in rat calvarial defects after surgical implantation of block or particulated biphasic calcium phosphate (BCP) lyophilized with *Escherichia coli*–derived recombinant human bone morphogenetic protein 2 (ErhBMP-2). Critical-size calvarial osteotomy defects were created in 5 groups of Sprague-Dawley rats. Each group received one of the following: 1) sham surgery control; 2) biphasic calcium phosphate particles (CPP); 3) biphasic calcium phosphate block (CPB); 4) ErhBMP-2–coated CPP; or 5) ErhBMP-2–coated CPB. ErhBMP was coated on BCP by a stepwise lyophilizing protocol. The new bone formation was significantly greater in ErhBMP-2–treated groups compared with the untreated group. In particular, the ErhBMP-2/CPB group showed stability of augmented areas during the period of healing, due to relevant space-providing capacity. Thus, it can be concluded that CPP and CPB lyophilized with ErhBMP-2 enhance the formation of new bone, and CPB appears to be a suitable carrier for ErhBMP-2 in which a 3-dimensional structural integrity is an important consideration factor. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;xx:xxx)**

Recombinant human bone morphogenetic proteins (rhBMPs), which are potent osteoinductors, have long been considered as a promising avenue for bone regeneration. Other bone substitutes have suffered from clinical limitations, such as long healing time, cost inefficiency, unsatisfactory bone regeneration, and noncompliance with the morphology of the defect. Although rhBMP-2 and rhBMP-7 (osteogenic protein-1) are commercially available for treatment of orthopedic and oral-maxillofacial defects,[1-3] rhBMP-2 is currently produced by mammalian cell cultures (e.g., Chinese hamster ovary cells) transfected with the BMP gene (CrhBMP-2), which is very costly, given the limited yield. One possible solution to this problem is to use rhBMPs derived from BMP gene–transfected *Escherichia coli* (ErhBMP-2), because this method can yield higher amounts of rhBMPs at relatively lower cost.

If rhBMP-2 is to be effectively deployed, another issue that should be considered is the carrier system. Like other growth factors, rhBMP-2 requires a delivery system that creates optimal conditions for cellular and vascular growth,[4-6] cellular attachment, and release kinetics. Calcium phosphates, such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), have been deemed to be suitable candidates for a rhBMP-2 delivery system, because of their space-providing properties. Moreover, as alloplastic materials, these ceramics are not only chemically and structurally similar to human bone tissue, but also have excellent osteoconductive capacity and proven biocompatibility.[7] Biphasic calcium phosphate (BCP) in particular, which is a specific ratio of HA and β-TCP, has well documented osteoconductive properties.[8-10] Practically, particle-type BCP is used for bone substitutes in dehiscence or small bony defects, whereas block-type BCP is used in large bony defects to provide space integrity and augment soft tissue for proper tissue shape. However, in many studies, rhBMP-2 has been loaded onto BCP moistened with a diluted solution of rhBMP-2 to produce implants, despite the inaccurate dose, uncontrolled flow, and inconvenient handling. Increased concerns about these problems have led to the acceptance of a method

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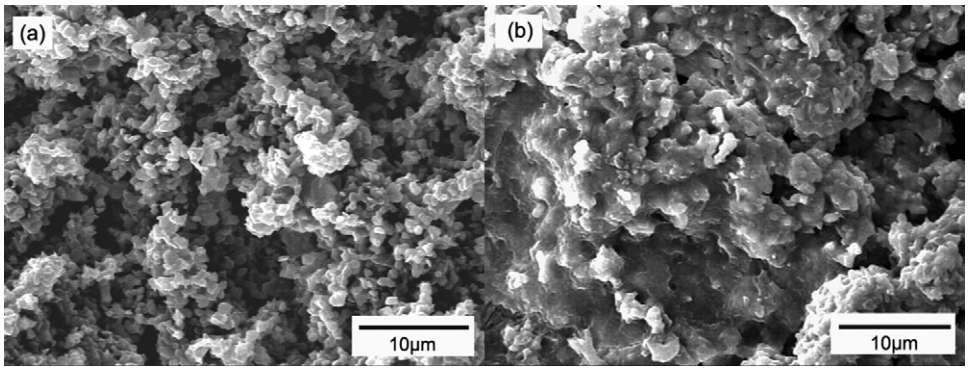

Fig. 1. SEM photomicrograph of ErhBMP-2 untreated BCP (**a**) and ErhBMP-2 coated BCP (**b**).

for loading rhBMP-2 onto biphasic calcium phosphate by coating it using a lyophilization protocol.

Until now, no study has demonstrated new bone formation with this method of coating BCP with ErhBMP-2. We have investigated the efficacy of bone formation in a rat calvarial defect after coating block or particulate BCP with ErhBMP-2.

MATERIAL AND METHODS

Animals

One hundred male Sprague-Dawley rats (body weight 250-300 g) were used. Rats were maintained in plastic cages in a room with an ambient temperature of 21°C, with 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 of Yonsei Medical Center, Seoul, Korea.

Materials

Expression of rhBMP-2 in E.coli. The ErhBMP-2 was produced at the research institute Cowellmedi Co., Pusan, Korea. A nonglycosylated rhBMP-2 was obtained in the form of inclusion bodies and was refolded in vitro into the active dimer form, as previously described.[11]

Preparation of calcium phosphate granules coated with rhBMP-2. Granules (0.5-1 mm in diameter) of microporous BCP (Bio-C; Cowellmedi) with a 30:70 ratio (HA/β-TCP) were used. The total porosity was ~70%. This was measured using the Brunauer-Emmett-Teller method with helium adsorption. Briefly, BCP granules were prepared by mixing calcium-deficient apatite with binder, making a granular form, and finally sintering at 1,100°C. The chemical purity of the BCP granules was analyzed by X-ray diffraction (D/MAX 2Rint 2700; Rigaku Co., Japan). The BCP granules were packed into a glass ampule and sterilized with γ-radiation.

E. coli–expressed rhBMP-2 solution (0.67 mL in 1.5 mg/mL buffer) was pipetted into an ampule containing 1 g of the BCP granules and lyophilized in a freezer-drier (Shinil, Co, Korea). The solution was frozen by placing the ampule on precooled shelves and cooling it down to –43°C. The formulations were maintained at this temperature for 3 hours, after which they were dried in a condenser at –40°C (primary drying) and kept in a pressure chamber at 5 mTorr for 2 hours. Secondary drying was performed on a shelf using the following sequence: –20°C for 4 hours, –10°C for 4 hours, 0°C for 2 hours, and 20°C for 20 hours. The chamber pressure was constant throughout the procedure.

Preparation of rhBMP-2–coated BCP blocks. To make a block form, we filled the premade hollow templates (inner diameter 8 mm, height 3 mm) with wet granules (diameter 0.2-1 mm) and pressed the granules flat with the back of a spatula, formed it into a block, and sintered it at 1,100°C. After preparation, surface morphology was observed by scanning electron microscopy (S-4,200; Hitachi, Japan) at 15 kV (Fig. 1).

Study design

The animals were divided into 5 groups of 20 animals each and were allowed to heal for 2 weeks (10 rats) or 8 weeks (10 rats). Each group received one of the following: 1) sham surgery; 2) BCP particle (CPP) alone; 3) BCP block (CPB) alone; 4) ErhBMP-2–coated CPP; or 5) ErhBMP-2–coated CPB.

Surgical procedures

The animals were anesthetized with an intramuscular injection (5 mg/kg body wt) of a 4:1 solution of ket-

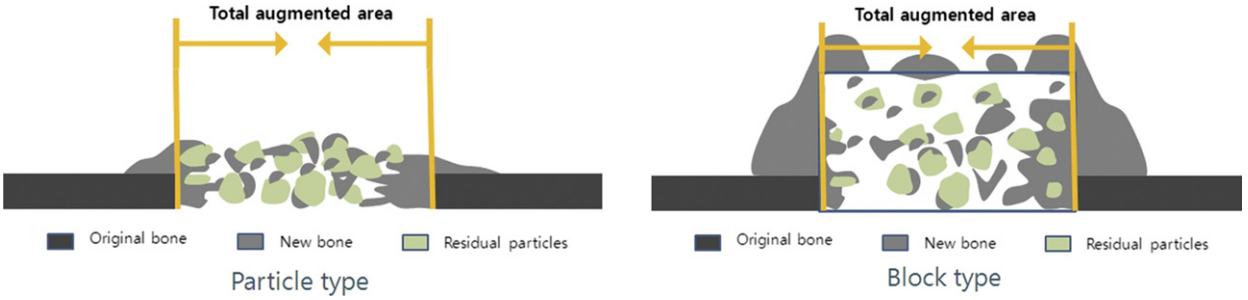

Fig. 2. Schematic drawing showing the histometric analysis. The bone density was calculated as new bone area per augmented area.

amine hydrochloride (Ketalar; Yuhan Co., Seoul, Korea) and xylazine (Rompun; Bayer Korea, Seoul, Korea). The surgical site was shaved and scrubbed with iodine. For the calvarial defect model, an incision was made in the sagittal plane across the cranium, and a full-thickness flap was reflected, exposing the calvarial bone. A standardized circular transosseous defect 8 mm in diameter was created on the cranium using a trephine drill (3i Implant Innovation; Palm Beach Gardens, FL, USA) with copious saline irrigation. After removal of the trephined calvarial disk, ErhBMP-2 or control treatments were applied to the defect sites. In the particulate ceramic groups, 0.05 g CPP or CPP/ErhBMP-2 (50 μg ErhBMP-2 per defect) was applied to the defect, whereas 0.25 g of CPB or CPB/ErhBMP-2 (250 μg E-rhBMP-2 per defect) was applied to the defect in the block ceramic groups. All surgical sites underwent primary closure using 4-0 Monosyn (glyconate absorbable monofilament; B-Braun; Aesculap, PA, USA).

Histologic processing

Block sections, including the surgical sites, were removed when the animals were killed. The sections were rinsed with sterile saline and fixed in 10% buffered formalin for 10 days. After being rinsed with water, the sections were decalcified in 5% formic acid for 14 days and embedded in paraffin. Serial sections of 5-μm thickness were cut through the center of the circular calvarial defects as well as the subcutaneous sites. From each block, 2 sections that contained the central portion were selected and stained with hematoxylin and eosin (H&E).

Analysis methods

Histologic analysis. The specimens was 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 as digital files.

Histometric analysis. After conventional microscopic examination, computer-assisted histometric measurements of the newly formed bone 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: augmented area, new bone area, and bone density. Only the area with newly formed mineralized bone was measured as new bone area (mm^2), and marrow and fibrovascular tissue were excluded. Bone density (%) was determined as the percentage of new bone area in the augmented area, meaning all tissues within the boundaries of the defects (mineralized bone, fibrovascular tissue, bone marrow, and residual biomaterials used as carrier; Fig. 2).

Statistical analysis

The statistical analysis was performed using commercially available software program (SPSS 15.0; SPSS, Chicago, IL, USA). Histomorphometric records from the calvarial defect samples were used to calculate the means (±SDs) of the group. The data were examined with the Kolmogorov-Smirnov test for conformance to a normal distribution.

A Kruskal-Wallis test was used to analyze the effects of time and experimental conditions. The post hoc Bonferroni test was used to analyze the difference between the groups ($P < .05$). A Mann-Whitney test was carried out to analyze the differences in parameters between the 2-week group and 8-week group.

RESULTS

Clinical observations

During the postoperative period, healing was uneventful for all animals. There were no complications, i.e., inflammatory reactions, exposure of graft material, or allergic reactions. Twelve specimens were excluded owing to technical complications during histologic processing (sham surgery control: 1 each at 2 and 8 weeks; CPP: 1 each at 2 and 8 weeks; CPP/ErhBMP-2: 1 at 2

Table I. Histometric results at 2 and 8 weeks, mm^2 (mean ± SD)

		CPP		*CPB*	
Parameter	*Sham (n = 18)*	*CPP alone (n = 18)*	*CPP/ErhBMP-2 (n = 19)*	*CPB alone (n = 16)*	*CPB/ErhBMP-2 (n = 17)*
2 wk					
Augmented area	0.80 ± 0.04	8.84 ± 2.68*	10.52 ± 2.45*	24.44 ± 3.19*	28.49 ± 2.32*†
New bone area	0.20 ± 0.04	2.27 ± 0.91*	7.55 ± 1.93*‡	2.18 ± 0.72*	10.95 ± 1.75*†
8 wk					
Augmented area	1.38 ± 0.22	9.90 ± 1.42*	14.35 ± 2.91*‡	19.44 ± 2.62*	26.13 ± 2.63*†
New bone area	0.34 ± 0.12	2.42 ± 0.75*	8.37 ± 1.68*‡	2.52 ± 1.03*	7.09 ± 1.73*†

CPP, biphasic calcium phosphate particles; *CPB,* biphasic calcium phosphate blocks; *ErhBMP-2,Escherichia coli* –derived recombinant human bone morphogenetic protein 2.
*Statistically significant difference compared with the surgical control group ($P < .05$).
†Statistically significant difference compared with the CPB-alone group ($P < .05$).
‡Statistically significant difference compared with the CPP-alone group ($P < .05$).

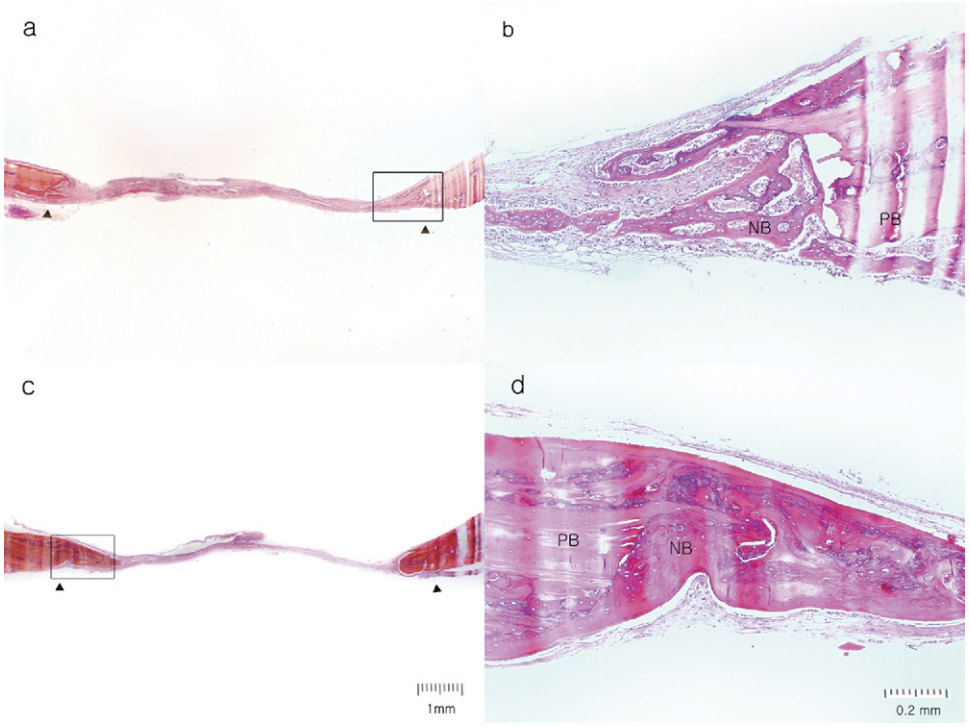

Fig. 3. Representative photomicrographs of the sham surgery group at 2 weeks (**a,b**) and 8 weeks (**c,d**). (Arrowheads indicate defect margin.) PB, pristine bone; NB, new bone. (H&E stain; original magnification:10x [a,c], 100x [b,d].)

weeks; CPB: 3 at 2 weeks and 1 at 8 weeks; and CPB/E-rhBMP-2: 1 at 2 weeks and 2 at 8 weeks). In the end, 88 specimens of the calvarial defect model were available for histomorphometric investigation (Table I).

Histologic observations

Sham surgery group. At 2 weeks, minimal amounts of new bone tissue had formed from the defect margins towards the central portion. Most of the area in a given defect was filled with a thin layer of fibrous connective tissue, which did not attain the thickness of the native calvarial bone. At 8 weeks after surgery, bone maturation had increased to a greater extent compared with after 2 weeks of healing (Fig. 3).

CPP group. At 2 weeks, the graft particles were well maintained under the connective tissue layer. New bone was seen at the periphery of the defect margin in the control specimens and around particles. The immature bone tissue was not only interconnected within itself, but it also partially encircled the graft particles (Fig. 4). At 8 weeks after surgery, progressive resorption of the graft materials was noticeable, and bone formation was

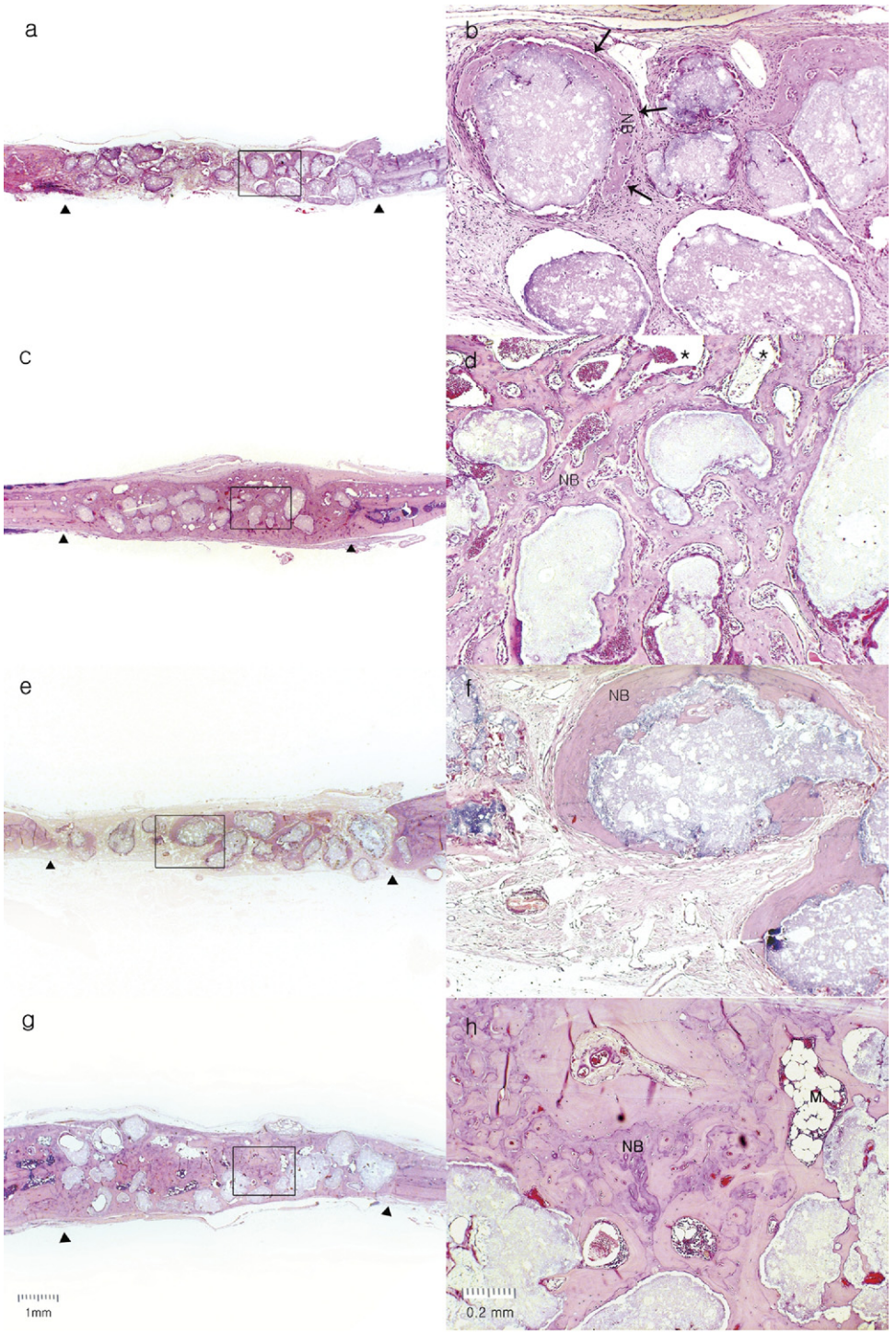

Fig. 4. Representative photomicrographs of the CPP groups at 2 weeks (**a,b**), 8 weeks (**e,f**) and the ErhBMP-2/CPP groups at 2 weeks (**c,d**), 8 weeks (**g,h**). Arrowheads indicate defect margin; arrows indicate osteoblastic cell lining. *, Blood vessel; NB, new bone. (H&E stain; original magnification:10x [a,c,e,g], 100x [b,d,f,h].)

increased compared with the 2-week specimens. The bony tissues around the particles were progressively interconnected (Fig. 4).

CPP/ErhBMP-2 group. At 2 weeks, a great amount of new bone formation was observed. The newly formed bone was lamellar type and had been depos-

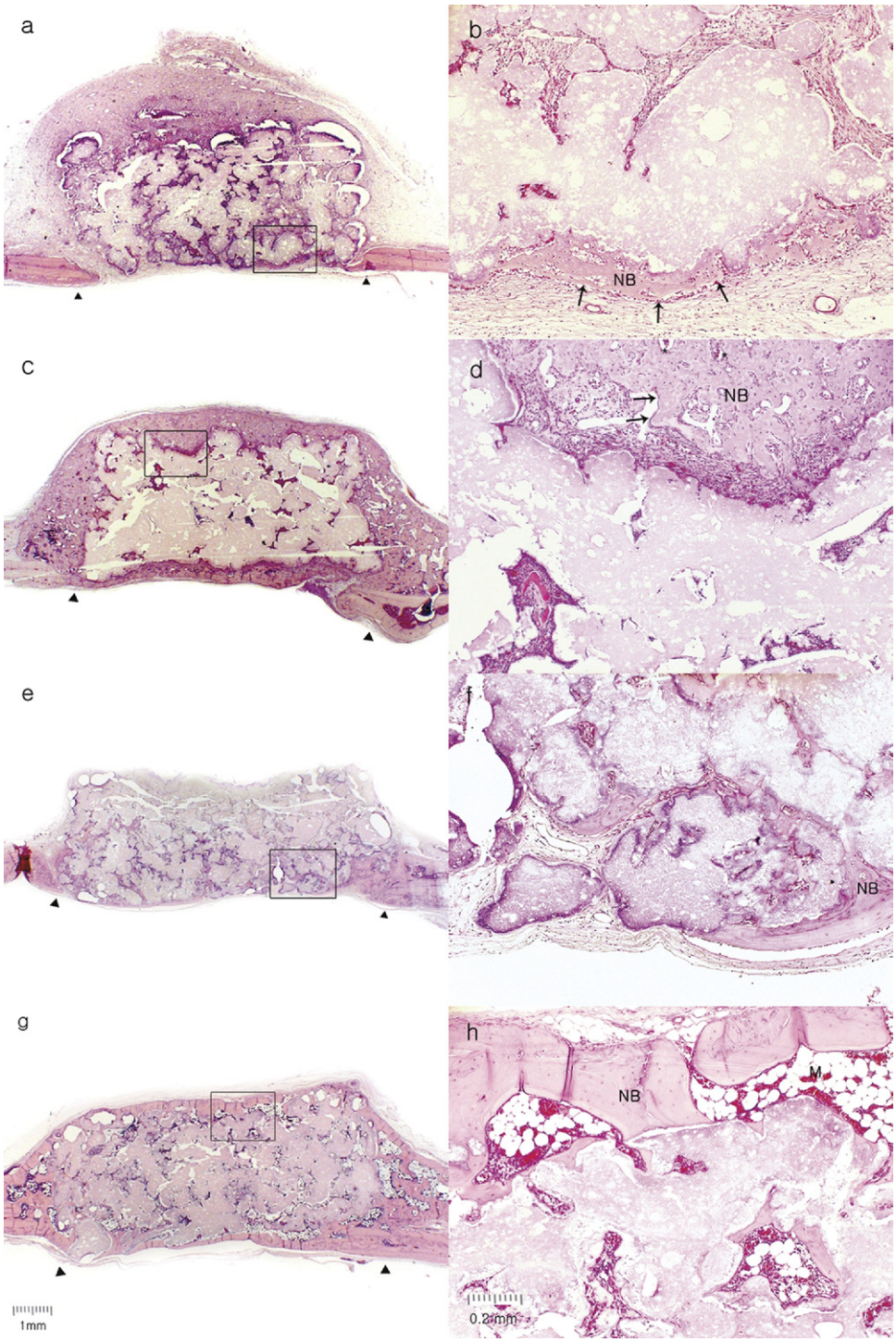

Fig. 5. Representative photomicrographs of the CPB groups at 2 weeks (**a,b**), 8 weeks (**e,f**) and the ErhBMP-2/CPB groups at 2 weeks (**c,d**), 8 weeks (**g,h**). Arrowheads indicate defect margin; arrows indicate osteoblastic cell lining. *, Blood vessel; NB, new bone. (H&E stain; original magnification:10x [a,c,e,g], 100x [b,d,f,h].)

ited directly onto the ceramic surface, extending randomly to form an anastomosing network of trabeculae. Osteocytes were visible in the lacunae, and osteoblasts were lined up along the bony trabeculae. At 8 weeks, newly formed bone had matured, and the increase in height had lessened, but the quantity of

Table II. Bone density (%, mean ± SD) at each time interval

		CPP		*CPB*	
	Sham	*CPP alone*	*CPP/ErhBMP-2*	*CPB alone*	*CPB/ErhBMP-2*
2 wk	25.98 ± 4.21	25.64 ± 8.61	71.66 ± 5.17*†	8.77 ± 1.94*	38.34 ± 4.26*‡
8 wk	24.31 ± 7.45	25.50 ± 10.92	58.47 ± 4.25*†	12.58 ± 3.54*	26.89 ± 4.85‡

Bone density = new bone area/augmented area × 100. Abbreviations as Table I.
*Statistically significant difference compared with the surgical control group ($P < .05$).
†Statistically significant difference compared with the CPP-alone group ($P < .05$).
‡Statistically significant difference compared with the CPB-alone group ($P < .05$).

the bone marrow had increased compared with the 2-week group (Fig. 4).

CPB group. At 2 weeks after surgery, the implanted block was surrounded by fibrous tissue infiltrated with inflammatory cells. The extent of bone induction was slightly greater than in the sham surgery group, and osteogenesis was observed on the side in contact with cranial bone, as well as on the inferior side of the block. This tendency was also observed 8 weeks after surgery, but more bone formation was observed in the pores of the block. New bone formation surrounding the block was not observed after 2- or 8-week healing intervals (Fig. 5).

CPB/E-rhBMP-2 group. As early as 2 weeks after defect creation, new bone had formed extensively around the blocks, and at 8 weeks newly formed bone was remodeled and diminished in quantity. At 8 weeks after surgery, the osseous union between the blocks and the cranium was strong, but the osteogenesis within the pores of the blocks was inadequate at both 2 and 8 weeks after insertion (Fig. 5).

Histometric observations

Histometric measurements are summarized in Tables I and II. Throughout our observation, the augmented area (mm^2) in the CPP and CPP/ErhBMP-2 groups was smaller than in the CPB and CPB/ErhBMP-2 groups. The area of newly formed bone was significantly greater in the ErhBMP-2–treated groups compared with the untreated group. We observed that the new bone area had increased in all groups by the end of the observation period with the exception of the CPB/ErhBMP-2 group.

At both 2 and 8 weeks, there were significant statistical differences in the extent of new bone area (mm^2) between the control group and other groups.

The new bone area was divided by the total augmented area; this relationship represents the bone density. For this parameter, all groups displayed similar results with new bone area, except for the CPP/ErhBMP-2 group.

DISCUSSION

The present study was designed to evaluate the efficacy of bone formation onto ErhBMP-2–coated HA/β-TCP particles and blocks by using an established rodent model with nonhealing calvarial defects. The findings indicate that ErhBMP-2–coated CPP and CPB promote new bone formation in a calvarial defect in rats. Even groups given CPP and CPB alone exhibited osteoconduction effects, representing direct contact between the new bone and the ceramics. These observations support earlier findings that bone formation is enhanced when rhBMP-2 is delivered by a synthetic matrix containing HA/β-TCP ceramics in a rat calvarial defect.[12] Collagen is the most documented carrier for rhBMP-2, but it is not osteoconductive and is less suited for onlay augmentation owing to its poor structural integrity.[5,13,14] Therefore, in the present study, we noted the space-providing properties of HA/β-TCP ceramics.

Earlier studies demonstrated that HA/β-TCP composites might have better bioactivity,[15,16] agreeing with other studies that revealed unpredictable bioresorption of HA or β-TCP alone. HA is poorly bioresorbable, whereas β-TCP undergoes gradual bioresorption.[17,18] These HA/β-TCP ceramics have already been investigated as to whether they can function as rhBMP-2 carriers, and loading rhBMP-2 onto HA/β-TCP has been found to induce favorable new bone formation.[12,19,20] However, the carrier was moistened with a dilution of rhBMP-2 in all of those studies. Although Alam et al.[15] and Jung et al.[12] loaded rhBMP-2 on HA/β-TCP by lyophilization, atelocollagen and polyethylene glycol were added to the ceramics as granule-binding media to facilitate plasticity and improve release kinetics. However, these additional procedures are inevitably accompanied by inconvenient manipulations. In the present study, the stepwise lyophilizing method from −40°C to 20°C was used to coat ErhBMP-2 onto ceramics for convenient storage and easy handling at room temperature without requiring additional procedures. Additionally, there is no need to

worry about inaccurate doses or uncontrolled flow with this method.

Both the CPP/ErhBMP-2 and CPB/ErhBMP-2 groups exhibited considerable new bone formation, similar to the results from earlier studies.[15] Furthermore, the total augmented area was stable during the healing periods in CPB/ErhBMP-2 groups. These results suggest that CPB could serve as a carrier system of ErhBMP-2 for bone tissue reconstruction in oral and maxillofacial areas, in which a 3-dimensional conformation and soft tissue compression during the healing periods are important consideration factors.

Besides the carrier system, the high cost of CrhBMP-2 has been another obstacle to its clinical use. ErhBMP-2 has been demonstrated to be biologically active in vitro[21] and in vivo,[22,23] despite the fact that ErhBMP-2 differs from CrhBMP-2 in that its molecular structure is missing 1 N-glycosylation. Additionally, Dohzono et al.[24] found that ErhBMP-2–adsorbed β-TCP granules could achieve posterolateral spinal fusion in a rabbit model as effectively as autogenous bone graft could. However, in their studies, ErhBMP-2 was dissolved in distilled water to reconstitute it for implant creation. Like the earlier authors, we found that implantation of ErhBMP-2–coated HA/β-TCP carriers facilitated new bone formation. The sham surgery control group produced fibrous connective tissue in most cases, although minimal bone formation was observed in areas close to the defect margins. The positive control groups for which ErhBMP-2 was not supplied produced restrictive bone formation, around particles in the CPP group and on the inferior side of the blocks in the CPB group.

In ErhBMP-2–treated groups, plenty of capillaries within the medullary space as well as lacunae containing osteoblasts were observed at 2 weeks, and the newly formed bone had remodeled such that volume was slightly reduced at 8 weeks. As previously reported, large quantities of BMPs may facilitate osteoclast formation and resultant bone resorption in a dose-dependent manner.[24,25] This excessive osteoclast differentiation and bone resorption is an indirect effect of osteoblast differentiation and receptor activator of nuclear factor kappa B ligand expression induced by BMP stimulation and is a direct effect of BMP on osteoclastogenesis.

In addition, unsatisfactory bone formation was observed inside the blocks in CPB/ErhBMP-2 animals. According to earlier investigators,[26] the progress of bone formation and resorption of grafted material was observed at 6 months after using biphasic calcium in a human sinus augmentation model. Although it is expected that new bone formation and bone remodeling would continue in the CPB/rhBMP-2 groups after 8 weeks, because osteoblastic cell lines and phagocytic cells were observed in macropores, further study about healing intervals and macroporosity is necessary.

Finally, ErhBMP-2 was coated onto ceramics in a single dose of 1 mg/mL per 1 g of HA/β-TCP ceramic, according to suggestions from earlier investigators.[27] In the context of the limited dose dependency of rhBMP-2,[28,29] a study to define the threshold dose is essential for minimizing systemic adverse effects.

CONCLUSIONS

Within the limitation of this study, it can be concluded that ErhBMP-2–coated CPP and CPB enhance the formation of new bone with easy handling for application, and the CPB carrier could be successfully used for onlay indication.

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