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ORIGINAL ARTICLE

TERM

Effects of Block Bone Substitutes Loaded with *Escherichia Coli*-Produced Recombinant Human Bone Morphogenetic Protein-2 on Space Maintenance and Bone Formation in Rat Calvarial Onlay Model

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We aimed to evaluate the effects of onlay-type grafted human freeze-dried corticocancellous bone block (FDBB) and deproteinized bovine bone with collagen (DBBC) loaded with *Escherichia coli*-produced recombinant human bone morphogenetic protein-2 (ErhBMP-2) on space maintenance and new bone formation in rat calvaria. Collagen sponge (CS), FDBB, or DBBC disks (8×4 mm) with ErhBMP-2 (2.5 μg) were implanted onto the calvaria of male Sprague-Dawley rats, whereas CS with buffer was implanted onto the calvaria as controls (n=20/carrier). Rats were killed at 2 or 8 weeks post-surgery for histologic and histomorphometric analyses; total augmented area, new bone area, and bone density were evaluated. At both time-points, all ErhBMP-2 groups showed significantly higher new bone area and bone density than the control group (p<0.05). ErhBMP-2/FDBB and ErhBMP-2/DBBC groups showed significantly higher total augmented area than ErhBMP-2/CS group (8 weeks), and ErhBMP-2/FDBB group showed significantly higher new bone area and bone density than ErhBMP-2/DBBC group (p<0.05). ErhBMP-2/CS group showed the highest bone density (p<0.05). Combining ErhBMP-2 with FDBB or DBBC could significantly improve onlay graft outcomes, by new bone formation and bone density increase. Moreover, onlay-grafted FDBB and DBBC with ErhBMP-2 could be an alternative to autogenous block onlay bone graft.

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Key Words: Freeze-dried corticocancellous bone block; Deproteinized bovine bone with collagen; *Escherichia coli*-produced recombinant human bone morphogenetic protein-2; Onlay graft; Space maintenance; New bone formation

INTRODUCTION

Sufficient bone width and height are the prerequisite for functional and esthetic implant. Horizontal and vertical bone augmentation of severely destroyed alveolar ridge would be necessary, and various biomaterials and surgical techniques have been developed for bone augmentation [1].

Autogenous bone grafts have been considered the gold standard in reconstructive surgery because of their bone regeneration mechanisms through osteogenesis, osteoinduction, and osteoconduction [2]. However, reconstruction with autogenous block onlay bone graft has shown clinical limitations such as donor site morbidity, limited volume of obtainable bone, increased time, the potential for complications, and pronounced resorption, especially in sites receiving mechanical load and soft tissue tension [3-5].

For these reasons, recent researches have been concentrating on the development and evaluation of allogenic or synthetic block type bone substitutes for onlay graft.

Allografts obtained from donors of the same species have biologic activity containing human mineralized component and collagen. The most common used form of allografts are frozen, freeze-dried, demineralizes freeze-dried, and irradiated. Freezing or freeze-drying the bone significantly reduced the antigenicity [6]. The freeze-dried bone allografts (FDBA) have been frequently applied for the periodontal and periimplant defects especially in particulated form. FDBA effectively enhanced space provision through long graft resorption time and induce induc-

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tive potential through growth factors stored in matrix [7].

Freeze-dried corticocancellous bone block (FDBB) has better mechanical stability compared to the particulated FDBA as well as osteoconductivity. Despite the successful results with FDBB in orthopedic surgery for decades, there have been only limited studies including case reports in alveolar ridge augmentation [8-11]. In our previous study, onlay grafts of human FDBB showed new bone formation in the rat calvarium through an osteoconduction. However, it appeared that *de novo* bone formation was limited [12].

Deproteinized bovine bone mineral (Bio-Oss®, Geistlich AG, Wolhusen, Switzerland) is a widely utilized commercial product in the form of cortical granules and well documented in periodontal and implant dentistry [13]. It is a natural matrix which is identical to the mineral phase of human bone. It has been reported to be highly osteoconductive and to have a very low resorption rate [14]. It has the macro- and micro-pore structure and the pores are of optimal size and configuration to facilitate vascular ingrowth, which is essential for new bone formation [15]. Thus, it was used as an efficient and affordable drug delivery systems [7]. However, despite the many advantages of deproteinized bovine bone mineral, it has also a shortcoming of particulated graft materials, i.e., difficulty in space maintenance without additional membrane.

Deproteinized bovine bone with collagen (DBBC) (Bio-Oss Collagen®, Geistlich AG, Woll-husen, Switzerland) consists of 90% of cancellous bovine bone granules and 10% of porcine collagen. Combining deproteinized bovine bone mineral with collagen enhances its handling characteristics making DBBC formable and easy to handle, and this give DBBC mechanical integrities. However, despite the many advantages of DBBC, xenogenic block grafts could be brittle and show low toughness when used in vertical bone augmentation [11]. It has shown that it served as a scaffold and preserved the ridge profile, but did not enhance the new bone formation [16]. Moreover, in our previous study the onlay grafted DBBC showed limited new bone formation, because the collagen of DBBC was absorbed after a few weeks and did not replace the barrier function of membrane [17]. Thus, it is necessary to combine an osteoinductive growth factor, such as bone morphogenetic protein (BMP) with FDBB and DBBC [18].

BMPs are a set of growth and differentiation factors acting on early osteoprogenitor cells so that they differentiate into mature osteoblasts. Several BMPs, including BMP-2, -4, -6 and -7, have been reported to have significant osteoinductive potential [19,20]. Among these, recombinant human BMP-2 (rhBMP-2) was found to have strong *in vivo* bone-inducing ability [20-22]. Previously, most rhBMPs have been produced in mammalian cells, such as the Chinese hamster ovary cells [23]. However, its low yield (ng/mL) and high cost produced in this eukaryotic protein expression system might be a limitation for clinical applications. Therefore various attempts have been made to evaluate biologically active rhBMPs in *Escherichia coli* (*E. coli*), as an alternative to mammalian cells. It has shown comparable biologic activity in comparison with rhBMP produced in a eukaryotic system [24-26], and would enable the high yield of rhBMPs at low cost.

However, despite plenty of BMP researches, the ideal carrier has not yet been found. Absorbable collagen sponge (CS) is one of the most frequently used carriers of BMPs, and its regenerative capacity has been identified in many researches [27]. However, the structural stability was uncertain in some animal and human studies [28,29], it becomes victim to compressive forces especially when used for non-space-providing onlay indications [25]. If the block type bone substitutes such as FDBB and DBBC are used for BMPs' carrier, we might expect that the cancellous portion of FDBB or the porous structure of DBBC could entrap the BMPs and release them slowly and, in addition, the rigid structure of them could provide resistance against compressive force.

Therefore, in this study, we used three carriers—CS, FDBB, and DBBC—loaded with *Escherichia coli*-produced recombinant human bone morphogenetic protein-2 in rat calvarium. The aims of the current study were to evaluate the effects of onlay type grafted FDBB and DBBC loaded with ErhBMP-2 on the space maintenance and new bone formation in the rat calvarium and to elucidate the efficacy of onlay grafts with FDBB and DBBC in point of eliminating the need for autogenous block onlay bone graft.

MATERIALS AND METHODS

Animals

Eighty male Sprague-Dawley rats (200−300 g) were used and maintained in cages in a room with 7-h day/night cycles, an ambient temperature of 21°C, and a standard laboratory pellet diet. All procedures were approved by the Institutional Animal Care and Use committee, Ewha Medical Center, Seoul, Korea (confirmation number: ESM 12-0188).

Materials

The expression of rhBMP-2 in *E. coli* was performed at the Research Institute of Cowellmedi Co. Ltd., Busan, Korea. Absorbable atelo CS (TERUPLUG®, Olympus Terumo Biomaterials, Tokyo, Japan) is cross-linked by heat treatment for biocompatibility. FDBB (AlloBone, Osteo.in, Seoul, Korea), and DBBC, a xenograft blended of granules of deproteinzed bovine bone (90%) and purified porcine collagen fibers (10%) (Bio-Oss®Collagen; Geistlich

Pharma AG, Wolhusen, Switzerland), is sterilized by gamma irradiation. All the materials were trimmed into disk shape with 8 mm in diameter and 4 mm in height (Fig. 1).

Surgical procedures

The animals were anesthetized by an intramuscular injection [0.1 mL/10 g of a 3:2 solution of tiletamine and zolazepam (Zoletil®, Virbac, TX, USA): xylazine HCl (Rumpun®, Bayer, Leverkusen, Germany)]. After full-thickness flap elevation, the recipient bed was subjected to six 1-mm wide monocortical perforations using the round burs. ErhBMP-2 (Cowellmedi Co., Ltd., Busan, Korea) was diluted in a solubilization buffer to concentrations of 0.025 mg/mL. Disk-shaped CS, FDBB, and DBBC were loaded with 0.1-mL ErhBMP-2 solution to produce implanted concentrations of 2.5 μg. For control, the CS was loaded with buffer alone. ErhBMP-2 was allowed to bind for 5 min, and implanted on the parietal bone and stabilized with horizontal mattress suture. The periosteum was replaced at the original site and sutured. The animals were divided into four groups, 1) control, 2) ErhBMP-2/CS, 3) ErhBMP-2/FDBB, and 4) ErhBMP-2/DBBC, of 20 animals each and allowed to heal for 2 (n=10) or 8 (n=10) weeks (Table 1, Fig. 2).

Histologic and histomorphometric analysis

The rats were sacrificed at 2 and 8 weeks after surgery. The specimens were harvested, fixed in 10% neutral formalin, decalcified and embedded in paraffin. They were sagittally sectioned in 4 μm thickness and stained with hematoxylin-eosin. The most central sections were evaluated. Computer-assisted histomorphometric measurements were obtained using an automated image analysis system (Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) coupled with a videocamera on a light microscope (Eclipse 50i, Nikon, Tokyo, Japan). Sections were examined at a magnification of 10× and 100×.

Histomorphometric parameters were defined as follows (Fig. 3):
Total augmented area (mm^2)=All tissues within the boundaries of newly formed bone including newly formed bone, residual graft materials, and fibrovascular tissues

· New bone area (mm^2)=Area of newly formed bone within the total augmented area

· Bone density (%)=Percentage of new bone area to the total augmentation area (New bone area/Total augmented area)×100.

Statistical analysis

Histomorphometric recordings from the samples were used to calculate means and standard deviations. For the comparisons among the treatment groups, Kruskal-Wallis test was used. Mann-Whitney U test was used for the comparison between 2 and 8 weeks recordings within each group. The post-hoc Scheffé's test was used to analyze the difference between the groups ($p<0.05$).

Table 1. Experimental groups

Group	No. of animals	
	2 weeks	8 weeks
Control (CS)	10	10
ErhBMP-2/CS	10	10
ErhBMP-2/FDBB	10	10
ErhBMP-2/DBBC	10	10

CS: collagen sponge, ErhBMP: *Escherichia coli*-produced recombinant human, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen

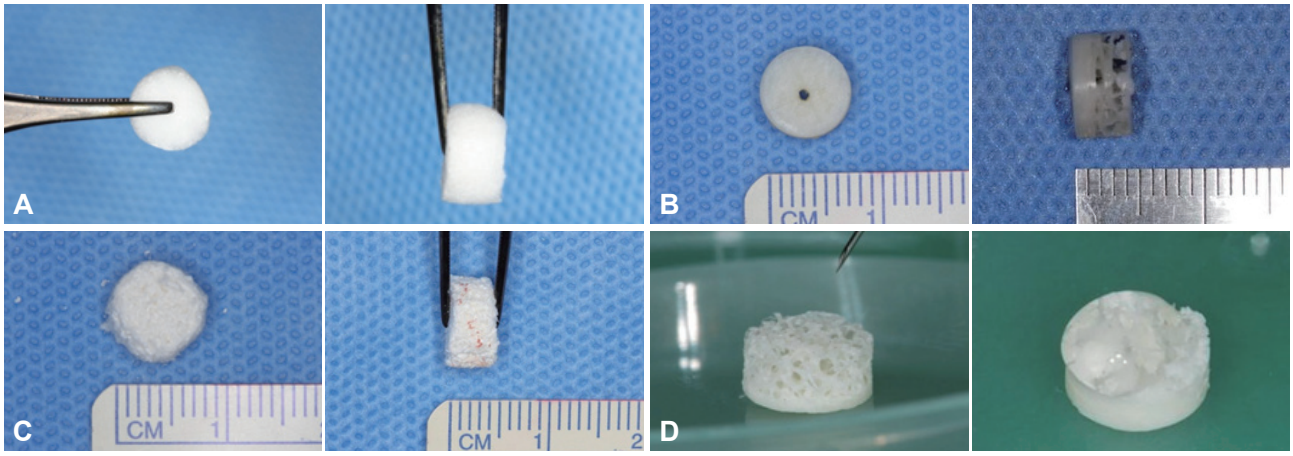

Figure 1. Preparation of graft materials (A) CS, (B) FDBB, (C) DBBC, (D) application of ErhBMP-2. CS: collagen sponge, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen, ErhBMP-2: escherichia coli-produced recombinant human bone morphogenetic protein-2.

RESULTS

Clinical observations

Wound healing was generally uneventful for all animals except one that belonged to the ErhBMP-2/CS group at 2 weeks and one that belonged to the ErhBMP-2/FDBB group at 8 weeks. These animals died for unknown reasons. No material exposure or other complications were observed at the surgical sites.

Histologic observations

Control groups

No suggestive image of new bone deposition was observed, and the volume of the graft material was collapsed prominently at 2 weeks (Fig. 4). At 8 weeks, limited bone formations were observed and volumes were collapsed more than at 2 weeks (Fig. 5).

ErhBMP-2/CS groups

At 2 weeks, newly formed immature bone was observed in the recipient-graft interface, and CS was much more resorbed, but still observed (Fig. 4). At 8 weeks, the CS was completely resorbed and almost replaced with the newly formed bone and the original bone was barely distinguishable from the new bone. In addition, the new bone had matured compared to that observed at 2 weeks. However, the graft volume was prominently collapsed at both 2 and 8 weeks (Fig. 5).

ErhBMP-2/FDBB groups

At 2 weeks, the osteogenesis was evident on the lateral margins of the grafted block and narrowly appeared on the central areas. The graft still appeared to be highly preserved (Fig. 4). At 8 weeks, the cancellous portion of the FDBB was considerably resorbed and the remodeling and osteogenesis process invaded the central region of the recipient-graft interface and intense bone formation adjacent to the graft and host bone could be observed. However, the cortical portion of the graft was not resorbed yet and the volume was still highly preserved. Newly formed bone was integrated into the original bone. For this reason, it was difficult to distinguish the location of the original defect margin (Fig. 5).

ErhBMP-2/DBBC groups

At 2 weeks, insignificant volume change of the graft material was appeared and newly formed immature bone was observed on the lateral margins of the grafted block as well as interface. A large number of residual DBBC particles were observed within the new bone (Fig. 4). Although the volume of the graft was slightly decreased, the grafted area was filled with newly formed bone at 8 weeks. The appearance of the new bone at 8 weeks was more lamellar than that at 2 weeks (Fig. 5).

Histomorphometric analysis

Twelve specimens were excluded due to technical complications in the histological processing. A total of 66 specimens were investigated histomorphometrically.

Total augmented area

The experimental groups showed significantly larger at both 2 and 8 weeks compared to the control group ($p<0.05$). All groups showed a significant decrease between 2 and 8 weeks ($p<0.05$). The ErhBMP-2/FDBB and ErhBMP-2/DBBC groups showed significantly larger at both 2 and 8 weeks compared to the ErhBMP-2/CS group ($p<0.05$), And the ErhBMP-2/DBBC group showed significantly larger at both 2 (56.75 ± 2.79 mm^2) and 8 weeks (43.99 ± 6.79 mm^2) compared to the ErhBMP-2/FDBB group (36.19 ± 3.00 mm^2 and 38.34 ± 1.73 mm^2, respectively) ($p<0.05$) (Table 2).

New bone area

The new bone area of the experimental groups showed significantly larger at both 2 and 8 weeks than that of the control group ($p<0.05$). It was significantly larger at 8 weeks than at 2 weeks ($p<0.05$). The ErhBMP-2/FDBB and ErhBMP-2/DBBC groups showed significantly larger at 8 weeks compared to the

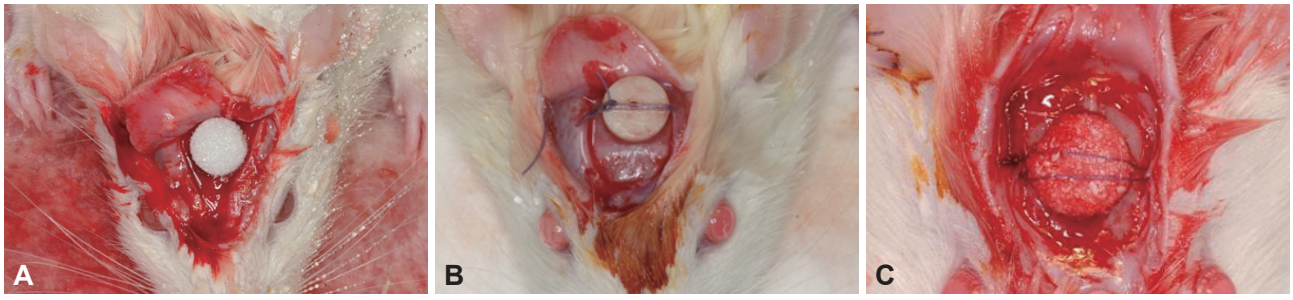

Figure 2. The graft materials were implanted onto the rat calvaria. (A) ErhBMP-2/CS, (B) ErhBMP-2/FDBB, (C) ErhBMP-2/DBBC. ErhBMP-2: *Escherichia coli*-produced recombinant human bone morphogenetic protein-2, CS: collagen sponge, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen.

ErhBMP-2/CS group (p<0.05). The ErhBMP-2/FDBB group showed significantly larger at both 2 (6.63±1.14 mm^2) and 8 weeks (21.74±4.57 mm^2) compared to the ErhBMP-2/DBBC group (4.65±1.09 mm^2 and 12.57±4.94 mm^2, respectively) (p<0.05) (Table 3).

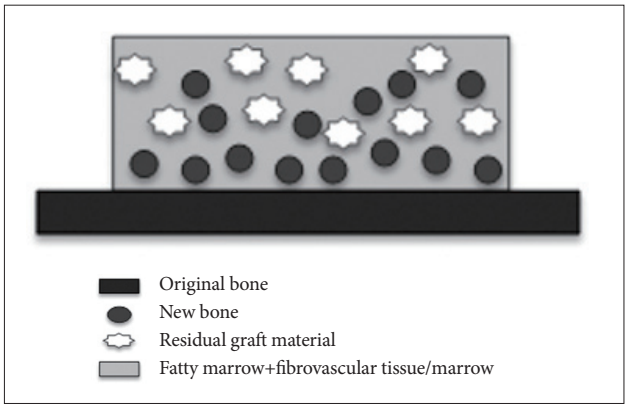

Figure 3. Schematic drawing to show the histomorphometric analysis of the calvarial onlay model.

Bone density

The experimental groups showed significantly higher bone density at both 2 and 8 weeks compared to the control (p<0.05). It was higher at 8 weeks than at 2 weeks (p<0.05). The ErhBMP-2/CS group showed significantly higher at 8 weeks compared to both the ErhBMP-2/FDBB and the ErhBMP-2/DBBC groups (p<0.05). The ErhBMP-2/FDBB group showed significantly higher at both 2 (18.38±3.21 mm^2) and 8 weeks (56.99±12.92 mm^2) than the ErhBMP-2/DBBC group (8.16±1.65 mm^2 and 27.89±6.83 mm^2) (p<0.05) (Table 4).

DISCUSSION

The onlay graft model was used in this study to evaluate the potential of graft materials for ridge augmentation. In case of inlay graft such as osseous defect, the graft materials are scarcely affected by biomechanics, so it is difficult to evaluate the effect of surrounding tissues. However, the materials implanted directly onto defect would receive pressure from the periosteum, mucosa, or skin, and the onlay graft model is considered to be

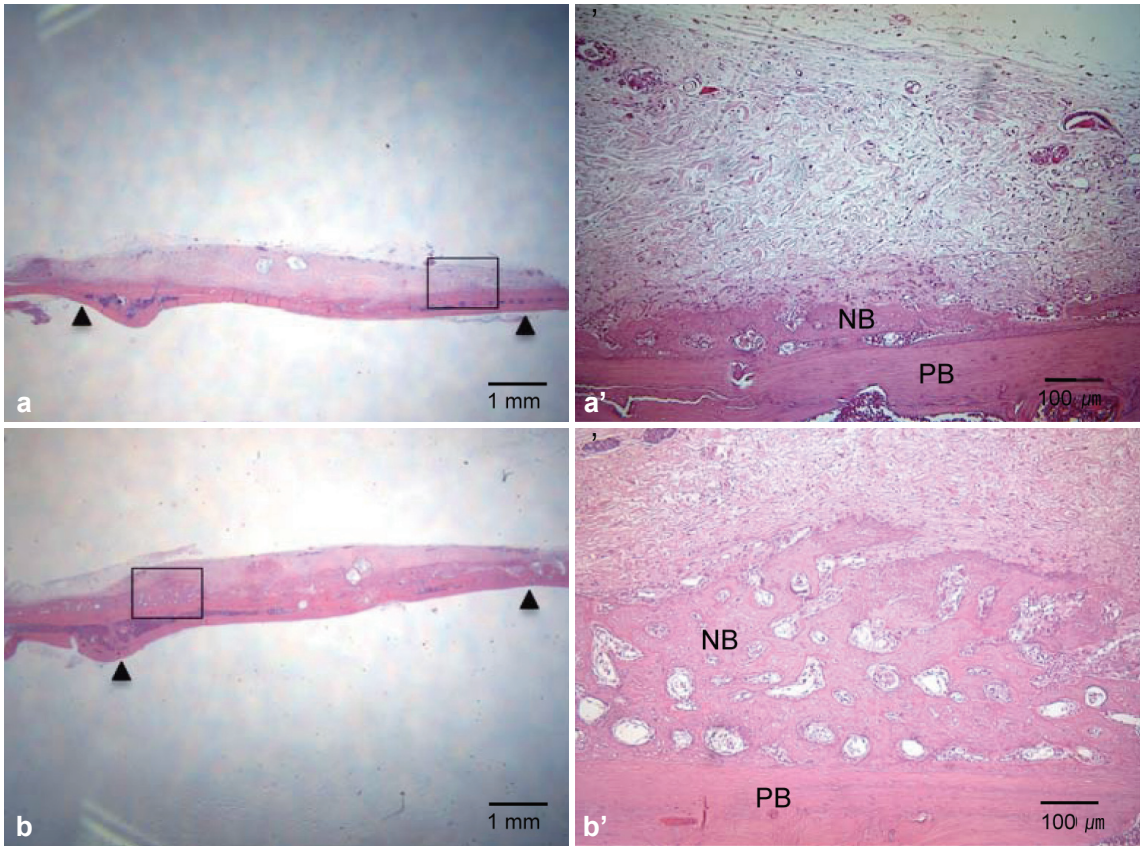

Figure 4. Representative photomicrographs of each group at 2 weeks. (a, a') control group, (b, b') ErhBMP-2/CS group, (c, c') ErhBMP-2/FDBB group, (d, d') ErhBMP-2/DBBC group (a', b', c', d') Region of interest (ROI) in a, b, c, d, arrowhead: grafted area margin. PB: pre-existing bone, NB: new bone, H-E stain, original magnification: a, b, c, d×10, a', b', c', d'×100. ErhBMP-2: *Escherichia coli*-produced recombinant human bone morphogenetic protein-2, CS: collagen sponge, BMP-2: bone morphogenetic protein-2, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen. (Continued to the next page)

more beneficial than inlay to evaluate the effectiveness for ridge augmentation [30].

BMPs are known to possess the ability not only to stimulate differentiation of uncommitted mesenchymal stem cells, but to enhance the differentiated function of osteoblasts [31]. We have previously investigated the effects of onlay grafts of FDBB and DBBC on bone formation in rat calvarium. These materials appeared to be effective in bone formation and followed the osteoconductive mechanism. However, it appears that new bone formation is limited owing to the lack of osteoinductivity [17]. In this study, ErhBMP-2 was combined with those materials for accelerating bone regeneration through osteoinduction. In our results, the experimental groups showed significantly larger new bone area at both 2 and 8 weeks compared to the control. These findings suggest that ErhBMP-2 might play an important role in the osteogenic mechanism as an osteoinductive molecule.

Many studies have attempted to determine the dose dependency of rhBMP-2, but this remains inconclusive. Kübler et al. [32] used 0.4, 4, and 40 mg doses of ErhBMP-2 and found a correlation between formation of new bone and ErhBMP-2 dose in ectopic implants. On the other hand, Lee et al. [31] investigated the efficacy of ErhBMP-2 doses of 2.5 mg or higher. They failed to show dose dependency in the rat calvarial defect model. Jang et al. [18] reported no dose dependency in the rat when the following doses of rhBMP-2 were used: 2.5, 5.0, 10.0, and 20.0 μg. Similarly, Pang et al. [33] found no dose dependency of rhBMP-4 in the rat calvarial defect model. Therefore, in this study, we used 2.5 μg doses of ErhBMP-2. Although the dose applied in this study may not be the minimum effective dose, we used it because low concentrations of ErhBMP-2 (2.5 μg) used in many previous studies did not show a significant dose-dependent response [33-36]. Future studies are needed to determine the minimum effective dose by using various doses lower than 2.5 μg.

We observed remarkable new bone formation in the ErhBMP-2-treated groups compared to the control. In our results, experimental groups at 8 weeks showed normal bone-forming process, including Haversian systems, cement lines, and marrow spaces.

Among the groups, the ErhBMP-2/FDBB group showed the most significant increase in new bone area and new bone formation at the recipient-graft interface. In fact, FDBB alone truly

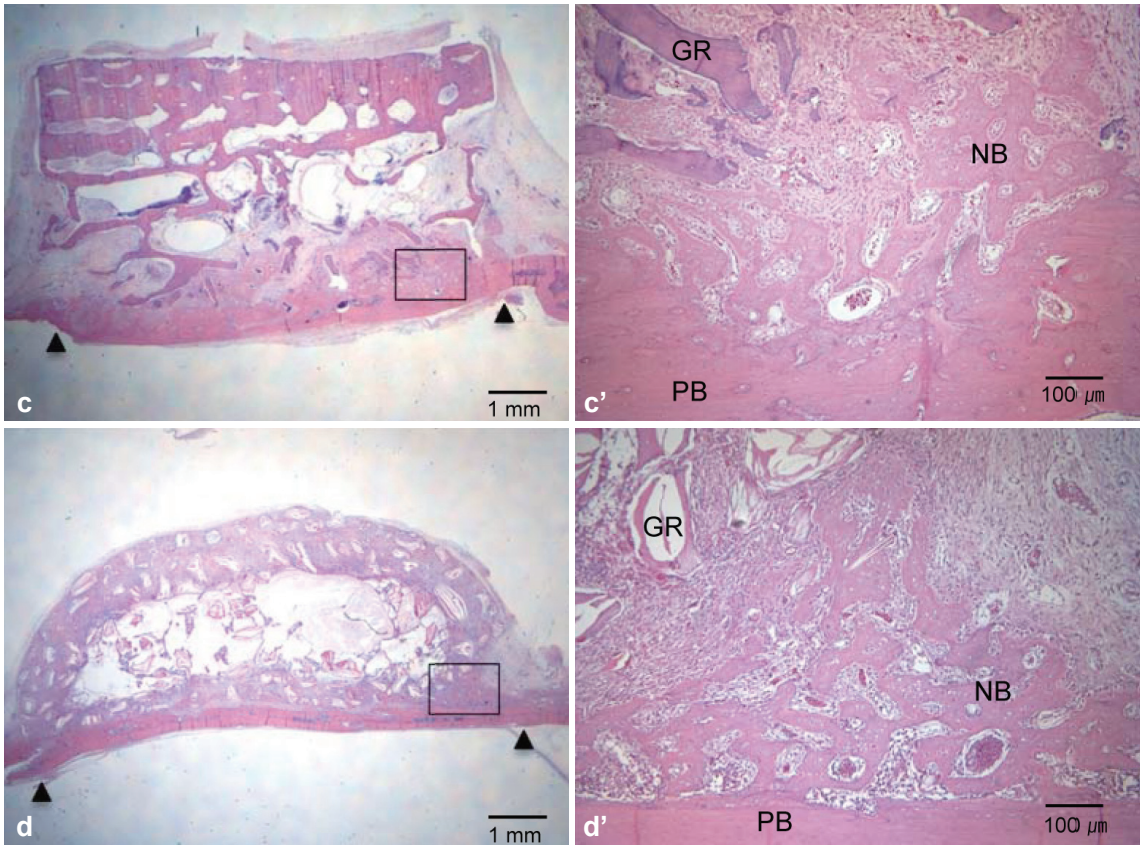

Figure 4. (Continued from the previous page) Representative photomicrographs of each group at 2 weeks. (a, a') control group, (b, b') ErhBMP-2/CS group, (c, c') ErhBMP-2/FDBB group, (d, d') ErhBMP-2/DBBC group (a', b', c', d') Region of interest (ROI) in a, b, c, d, arrow head: grafted area margin. PB: pre-existing bone, NB: new bone, H-E stain, original magnification: a, b, c, d×10, a', b', c', d'×100. ErhBMP-2: *Escherichia coli*-produced recombinant human bone morphogenetic protein-2, CS: collagen sponge, BMP-2: bone morphogenetic protein-2, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen.

act as an osteoconductive scaffold by stabilizing the blood clot and facilitating the cell ingrowth.

FDBA can be used in either a mineralized (FDBA) or demineralized (DFDBA) form. Demineralization process removes the mineral phase of the graft material and purportedly exposes the underlying bone collagen and possibly some growth factors, particularly bone morphogenetic collagen (BMP), which may increase its osteoinductive capacities [37]. FDBA may form bone by osteoconduction, and it hardens faster than DFDBA because it is mineralized. However, a previous study evaluating FDBA to DFDBA revealed no statistical difference in periodontal bone gain between the two allografts [38]. Because demineralization process was not performed on FDBB, it is likely that FDBB could be accelerating new bone formation mainly by osteoconduction

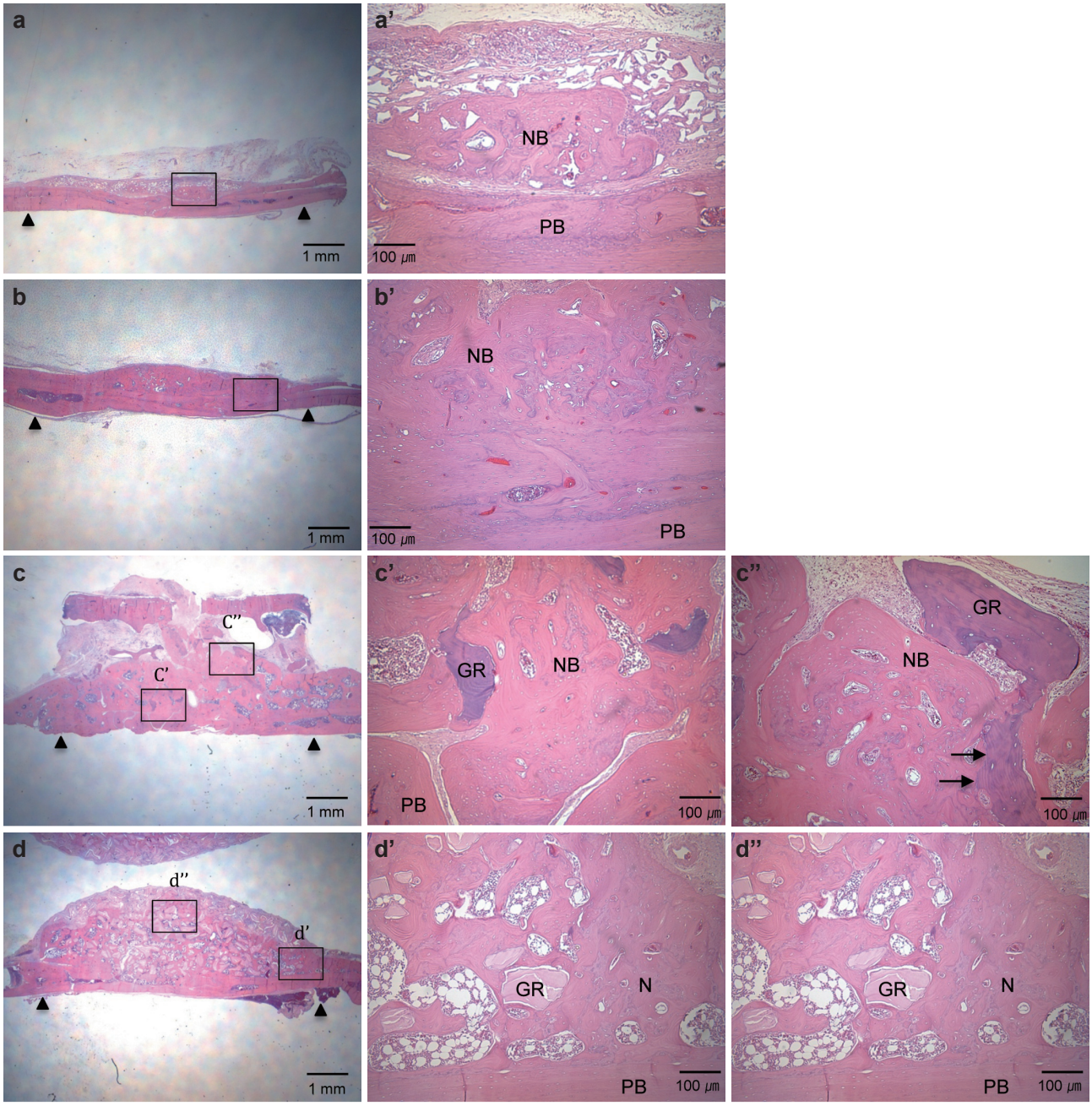

Figure 5. Representative photomicrographs of each group at 8 weeks. (a, a') control group, (b, b') ErhBMP-2/CS group, (c, c', c") ErhBMP-2/FDBB group, (d, d', d") ErhBMP-2/DBBC group (a', b', c', c", d', d"). Region of interest (ROI) in a, b, c, d, arrowhead: grafted area margin. PB: pre-existing bone, NB: new bone, H-E stain, original magnification: a, b, c, d×10, a', b', c', c", d', d"×100. ErhBMP-2: *Escherichia coli*-produced recombinant human bone morphogenetic protein-2, CS: collagen sponge, BMP-2: bone morphogenetic protein-2, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen.

without intrinsic potential of osteogenesis and osteoinduction and act as carriers for growth factors and morphogens. Therefore, the combination of the FDBB with the ErhBMP-2 has generated the osteogenic potential to stimulate osteoblast differentiation and vascular proliferation.

Furthermore, in contrast to the control and the ErhBMP-2/CS group the volume of the FDBB appeared to be highly preserved at 2 and 8 weeks. The increased cortical micro-architecture of the FDBB might have preserved the original bone volume over time [39,40]. We assumed that the cortical portion of the FDBBs allowed for mechanical stability, while the cancellous portion induced vascular infiltration and subsequent integration. In comparison with absorbable collagen carrier, block type allogeneic graft carrier (FDBB) exhibited remarkable space maintenance and enhanced new bone formation in this study. The tensional force or pressure may jeopardize the new bone formation and newly formed bone may be vulnerable by the force in the collagen carrier [41]. Thoma et al. [42] had a similar finding that the rhBMP-2 with block allograft provided the greatest ridge width compared to absorbable CS under a titanium mesh, in a lateral augmentation of the mandibular canine area. These indicated that the optimal delivery of BMPs required the structural stability which could be beneficial in facilitating osteoinductive effects.

Table 2. Total augmented area

Group	n	2 weeks	n	8 weeks
Control (CS)	9	7.58±1.53	8	5.49±1.35§
ErhBMP-2/CS	7	23.44±1.35*	9	17.19±1.73*§
ErhBMP-2/FDBB	9	36.19±3.00*†	9	38.34±1.73*†§
ErhBMP-2/DBBC	6	56.75±2.79*†‡	9	43.99±6.79*†‡§

Mean±standard deviation; mm^2, n=number of specimens. *statistically significant difference compared to control group ($p<0.05$), †statistically significant difference compared to ErhBMP-2/CS group ($p<0.05$), ‡statistically significant difference compared to ErhBMP-2/FDBB group ($p<0.05$), §statistically significant difference compared to 2 weeks group ($p<0.05$). CS: collagen sponge, ErhBMP: *Escherichia coli*-produced recombinant human, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen

Table 3. New bone area

Group	n	2 weeks	n	8 weeks
Control (CS)	9	0.08±0.08	8	0.16±0.10
ErhBMP-2/CS	7	5.04±0.68*	9	13.39±1.53*§
ErhBMP-2/FDBB	9	6.63±1.14*†‡	9	21.74±4.57*†‡§
ErhBMP-2/DBBC	6	4.65±1.09*	9	12.57±4.94*†§

Mean±standard deviation; mm^2, n=number of specimens. *statistically significant difference compared to control group ($p<0.05$), †statistically significant difference compared to ErhBMP-2/CS group ($p<0.05$), ‡statistically significant difference compared to ErhBMP-2/DBBC group ($p<0.05$), §statistically significant difference compared to 2 weeks group ($p<0.05$). CS: collagen sponge, ErhBMP: *Escherichia coli*-produced recombinant human, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen

Table 4. Bone density

Group	n	2 weeks	n	8 weeks
Control	9	0.95±0.96	8	2.65±1.18§
ErhBMP-2/CS	7	21.57±3.06*‡	9	77.78±1.64*†‡§
ErhBMP-2/FDBB	9	18.38±3.21*‡	9	56.99±12.92*‡§
ErhBMP-2/DBBC	6	8.16±1.65*	9	27.89±6.83*§

Mean±standard deviation; %, n=number of specimens. *statistically significant difference compared to control group ($p<0.05$), †statistically significant difference compared to ErhBMP-2/FDBB group ($p<0.05$), ‡statistically significant difference compared to ErhBMP-2/DBBC group ($p<0.05$), §statistically significant difference compared to 2 weeks group ($p<0.05$). CS: collagen sponge, ErhBMP: *Escherichia coli*-produced recombinant human, FDBB: freeze-dried corticocancellous bone block, DBBC: deproteinized bovine bone with collagen

In the ErhBMP-2/DBBC group, newly formed bone was found not only at recipient-graft interface but also in the augmentation area far from the original bone, whereas new bone formation was shown mainly at the recipient-graft interface in the ErhBMP-2/FDBB group. We assumed that the collagen within the DBBC was able to hold the ErhBMP-2 and release it in the early stage of healing. Furthermore, the pores in the DBBC which are of optimal size and configuration could facilitate vascular ingrowth [15], and entrap the ErhBMP-2 to make its prolonged diffusion possible. In this way, DBBC could make the controlled release of the ErhBMP-2 possible. The porous structure allows cells and newly forming tissues to migrate into it, so provides new bone formation and sufficient firmness against soft tissue pressure [33].

In our results of the ErhBMP-2/DBBC group, the total augmented area of appeared to be highly preserved at 2 weeks (56.75 mm^2) but significantly reduced at 8 weeks (43.99 mm^2). This may be because the porcine collagen which was included in the DBBC expanded rapidly in the initial stage of healing but underwent gradual degradation over time, particularly in the center of the DBBC (Fig. 4). Nonetheless, the total augmented area of the ErhBMP-2/DBBC group was significantly higher at 8 weeks compared to that in the ErhBMP-2/CS and the ErhBMP-2/FDBB groups ($p<0.05$). Previous studies have described that collagen coated alloplastic grafts facilitate the bone generation [43]. They interpreted that the role of collagen was to imbibe the blood easily and make the matrix formation reliable. Similarly, in our result, the addition of collagen in DBBC could benefit the bone regeneration in the same way.

Furthermore, the porous structure allows cells and newly forming tissues to migrate into it, so provides new bone formation and

sufficient firmness against soft tissue pressure [33]. The slowly resorbed demineralized bone particles has remained and attributed to the maintenance of augmented volume. The resorption time of deproteinized bovine bone matrix is very slow. The particles remained intact for 3–6 months and were remodeled during 18–24 months. Therefore during that time they could act as a scaffold for inducing bone formation via osteoconductive mechanism. In this way, the slow resorption rate could be beneficial to maintain the space during the early phase of bone formation. However, there is a growing controversy whether the prolonged resorption time has any clinical or histological adverse effect. Nevertheless, when the bone substitute was used as the carrier of a growth factor, the resorption process might be accelerated because the grow factor promote the bone formation rate [44], and the resorption process did not interrupt bone formation and maturation as well [44].

Various carriers have been investigated but the ideal carrier is yet to be determined. CS is one of the most widely used carriers [45-47]. Although it has remarkable bone formation capacity, it didn't have enough mechanical strength against the compressive forces from flap and lip [33,48]. Thus, the use of DBBC as a carrier could have the strengths of CS, and compensate the weaknesses of CS as well.

Even though the total augmented area of the ErhBMP-2/DBBC group was significantly higher than that of the ErhBMP-2/FDBB groups ($p<0.05$), the shape of the augmented bone of the ErhBMP-2/DBBC group was rounded at the edge of the DBBC. On the other hand the shape of the augmented bone of the ErhBMP-2/FDBB group was highly preserved. We assumed that it was due to the fast resorption of the collagen. Therefore, further studies using additional membranes are needed to redeem the shortcomings.

Bone density was significantly higher in the ErhBMP-2/CS group compared to the other groups. In fact, the amount of new bone area was similar to that of the ErhBMP-2/DBBC group, so, the greatest density might be attributed to its smallest total augmentation area. Although CS may be considered an effective carrier for ErhBMP-2, it is vulnerable to compressive forces when used for onlay indications [33,42,45]. Jung et al. [49] also reported that the collagen carrier did not fulfill the expectations of maintaining space for bone formation by ErhBMP-2. Furthermore, in terms of releasing of BMPs, CS and graft materials have shown different features that graft materials revealed sustainable releasing of BMPs as compare with collagen [50].

In this study, we could only examine early stage effects because of the short experimental periods. Therefore, further studies are required to clarify the effect of ErhBMP-2 on the later stages to elucidate the precise role of ErhBMP-2 during bone maturation.

Conclusions

Within the limits of the present study, it was concluded that outcomes of onlay graft are significantly improved, especially in terms of new bone formation and space maintenance, when ErhBMP-2 is combined with FDBB, or DBBC. FDBB and DBBC can be one of the effective carriers of ErhBMP-2, and the onlay grafts with FDBB and DBBC may be an alternative to autogenous block onlay bone grafts for the reconstruction of severely destroyed alveolar ridge.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Institutional Animal Care and Use committee, Ewha Medical Center, Seoul, Korea (confirmation number: ESM 12-0188).

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