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ORIGINAL ARTICLE

TERM

Histomorphometric Evaluation of Onlay Freeze-Dried Block Bone and Deproteinized Bovine Bone with Collagen in Rat

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The aim of this study was to evaluate the effect of human freeze-dried bone block (FDBB) and deproteinized bovine bone with collagen (DBBC) on bone formation when applied as an onlay graft in rat calvariums. Thirty male Sprague-Dawley rats received collagen sponge (control), FDBB, or DBBC onlay grafts trimmed into 8-mm disks measuring 4-mm height. Each graft was secured onto the calvarium surface using horizontal mattress sutures. Rats in each group were killed at 2 (n=5) or 8 (n=5) weeks postoperatively for histologic and histomorphometric analysis. The total augmented area (mm^2), new bone area (mm^2), and bone density (%) were measured. The FDBB and DBBC groups showed significantly more new bone formation and bone density than the control group at 2 and 8 weeks. The increased new bone area was significantly greater in the FDBB group than in the DBBC group ($p<0.05$). The total augmented area was significantly higher in the FDBB and DBBC groups at 2 and 8 weeks than in the control group ($p<0.05$), and at 8 weeks, the area was significantly decreased in the DBBC group compared to that in the FDBB group and the area at 2 weeks ($p<0.05$). Within the limitations of the present study, we concluded that onlay FDBB and DBBC grafts caused new bone formation through an osteoconductive mechanism. In addition, compared to FDBB, DBBC had less capacity to form new bone and maintain the space. Tissue Eng Regen Med 2016;13(1):70-77

Key Words: Human freeze dried corticocancellous bone block; Deproteinized bovine bone with collagen; Onlay graft; Bone formation; Space maintenance

INTRODUCTION

Augmentation of the alveolar bone has gained attention in the field of dental surgery, and many studies have investigated methods for restoration of resorbed alveolar bone following tooth extraction, especially as dental implants have become increasingly common. One frequently used alveolar bone augmentation technique is bone grafting with autogenous bone or bone substitute [1-3]. Among the multiple factors considered in bone grafting procedures, the particular type of bone graft material is one of the important elements determining clinical success. Autogenous bone is the gold standard and first choice clinically and has long been considered the most stable graft material with superior ability to trigger bone formation [4]. However, there are several concerns in autogenous bone grafting, it requires a second donor site, carries an increased surgical time and cost, and may be limited by the volume of obtainable bone [5-7]. Therefore, development and advancements in artificial graft materials lacking these limitations as a replacement of autogenous bone are being actively pursued.

Several studies and clinical reports of allogenic bone graft materials, such as human freeze-dried bone allograft and human demineralized freeze-dried bone allograft (DFBA), and xenogenic bone graft materials, such as deproteinized bovine bone (DBB) and deproteinized bovine bone with collagen (DBBC), have found that these as graft materials can replace autogenous bone [8-10]. Recently, allogenic graft materials that were fabricated into block form have been evaluated for the restoration of vertical height in patients with severe resorption of the alveolar ridge. These studies were intended to address the limitations of particle-type graft materials, which are especially susceptible to deformation caused by stress that de-

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velops within the tissue during postoperative healing [11,12]. There is a need to prevent collapse of the graft material and maintain the space for bone regeneration by using additional materials such titanium mesh or a titanium-reinforced barrier membrane [13].

Several clinical studies confirmed the predictability of block-type allografts, and found that a tight bone fusion could be established at the onlay bone-recipient interface [13-15]. In addition to these benefits, allogenic graft materials also carry the risk of infection and may cause non-uniform bone formation depending on the supplying bone bank [16]; as a result, xenograft and alloplast are being actively investigated as alternatives to overcome these disadvantages. Among them, deproteinized bovine graft material (Bio-Oss® collagen, Geistlich Pharma AB, Wolhusen, Switzerland), which has a bony trabecula that closely resembles the bone in humans, is one of the most widely used bone substitutes compared with other xenogenic bones. It reportedly has effective osteoconductivity, and many recent studies of DBBC have added 10% collagen to the grafted bovine bone to provide shape [17,18].

Nevins et al. [19] reported that owing to its superior ability to maintain the alveolar space, DBBC can be used without a barrier membrane for intrabony defects in relatively good shape; they also found that its particle adhesion to the damaged area was outstanding. Sculean et al. [20] demonstrated how easily DBBC can be applied clinically as the collagen fibers maintains the shape of the tissue by holding the particles together. Most of those studies were performed in periodontal defects, extraction sockets, and bony dehiscence, in which structural integrity was preserved and space was maintained for bone regeneration [21,22]. However, there are no reports describing DBBC grafting in onlay form, and histological and histomorphometric analysis studies are lacking.

Therefore, in this report, the bone formation and space maintenance in human freeze-dried bone and DBBC grafted in block form onto rat calvarium were comparatively analyzed using histological and histomorphometric analyses.

MATERIALS AND METHODS

Experimental animals

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

Materials

Absorbable atelo CS (TERUPLUG®; Olympus Terumo Biomaterials, Tokyo, Japan) consists of 85−95% collagen type I and 5−15% collagen type III, and is cross-linked by heat treatment for biocompatibility. Human freeze-dried bone block (FDBB) (AlloBone; Osteo.in, Seoul, Korea), which is sterilised by low dose gamma irradiation, was used. It was composed of ourter cortical and inner cancellous bone. DBBC (Bio-Oss® Collagen; Geistlich Pharma AG, Wolhusen, Switzerland) consists of cancellous bovine bone granules with the addition of 10% purified porcine collagen, and is sterilised by gamma irradiation. It is served as a matrix consisting of interconnection with macro and micropores (250 to 450 μm). The calcium components were varied in 38−42% and the phosphorus were in 12.5−17.5%. 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 anesthetised by an intramuscular injection [0.1 mL/10 g of a 3:2 solution of Zoletil® (Virbac, Carros, France): xylazine (Rompun, Bayer Korea, Seoul, Korea)]. Full-thickness flap was reflected, thus exposing the calvarial bone. With the use of round burs, the recipient bed was subjected to six 1-mm wide monocortical perforations. Then the material was implanted on the parietal bone and stabilised with horizontal mattress suture, and for control experiments, the CS was im-

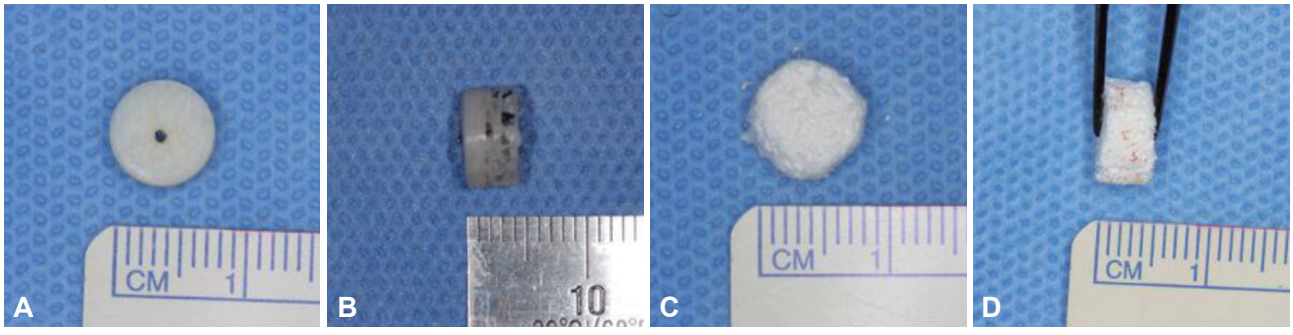

Figure 1. Preparation of graft materials. (A and B) Freeze-dried bone block group. (C and D) Deproteinized bovine bone with collagen group.

planted. After implantation, the periosteum was replaced at the original site. The animals were divided into three groups of 10 animals each and allowed to heal for 2 (n=5) or 8 (n=5) weeks. Each animal was assigned to one of three experimental groups: 1) control, 2) FDBB, and 3) DBBC (Table 1, Fig. 2).

Histologic and histomorphometric analysis

The rats were sacrificed by CO_2 asphyxiation at 2 and 8 weeks after surgery. Tissue blocks including the materials were harvested, fixed in 10% neutral formalin, decalcified and embedded in paraffin. The specimens were sagittally sectioned to about 4 μm in thickness and stained with haematoxylin-eosin. The most central sections from each block were selected for the histologic evaluation. 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.

Table 1. Experimental design

Groups	No. of experimental animals	
	2 weeks	8 weeks
CS	5	5
FDBB	5	5
DBBC	5	5

CS: collagen sponge, FDBB: freeze-dried bone block, DBBC: deproteinized bovine bone with collagen

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 Bonferroni correction was used to analyse the difference between the groups ($p<0.05$).

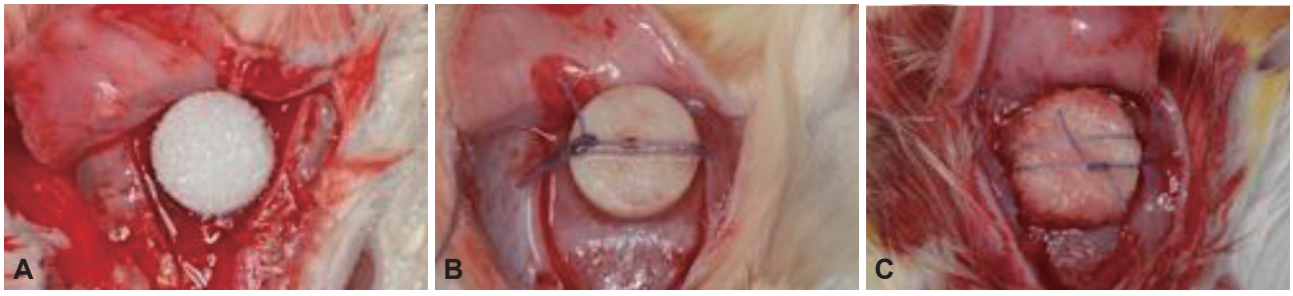

Figure 2. Grafting procedures in rat calvarium. (A) Collagen sponge. (B) Freeze-dried bone block. (C) Deproteinized bovine bone with collagen.

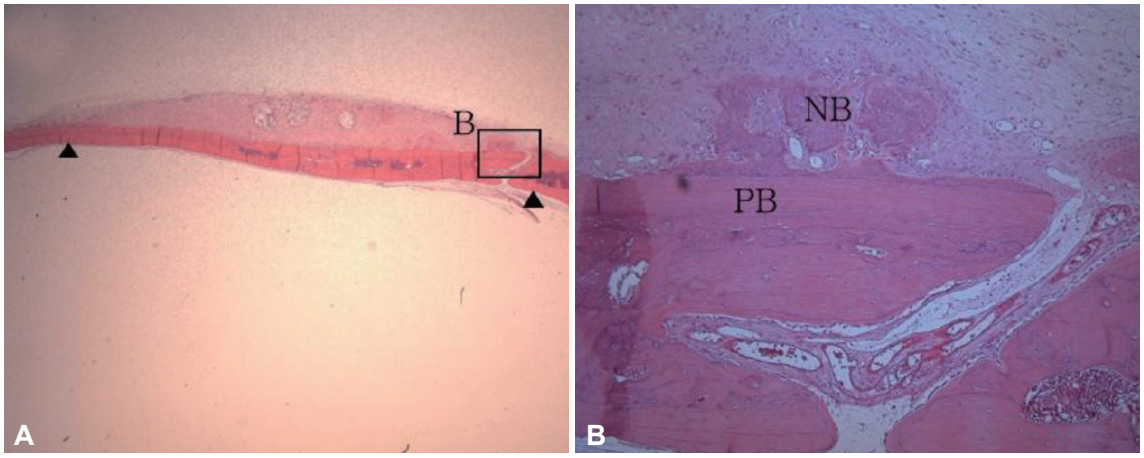

Figure 3. Representative photomicrographs of collagen sponge group at 2 weeks (B: region of interest in A; arrowhead: grafted area margin, H-E stain; original magnification: A ×10; B ×100). PB: pre-existing bone, NB: new bone.

RESULTS

Clinical observation

Clinically, the surgical sites healed well, and no adverse events occurred in any group. None of the animals exhibited graft exposure at the surgical site, inflammation, or other complications.

Histologic analysis

In the control group, new bone formation was largely absent during the 2-week postoperative period, and the volume and shape of the graft area collapsed significantly, with some unabsorbed collagen observed (Fig. 3). The collapse worsened slightly beyond week 2, but the change was not significant. Very minimal new bone formation was observed in the region bordering the existing bone (Fig. 4).

In the FDBB group, the overall shape of the graft material was well maintained at week 2 postoperatively (Fig. 5A). Multiple areas of new bone formation were observed along the edges of the graft area, and limited bone formation was observed centrally (Fig. 5B). At week 8, though the overall shape of the graft material decreased minimally, there was increased resorption of the adjacent spongy bone (Fig. 6A). Tight bone formation was observed along the border region between the existing bone and graft material (Fig. 6B), and new bone reached the central graft area (Fig. 6C).

In the DBBC group, minimal graft resorption was observed at week 2 postoperatively, and the overall volume was maintained, but the upper margins of the graft were dome-shaped due to collapse of the graft edges (Fig. 7A). A small amount of bone formation was observed at the interface between the graft edges and recipient site, central area (Fig. 7B), and further from the border region. Rather than new bone, residual bone particles were detected within the connective tissues (Fig. 7C). At week 8, compared to week 2, the graft material exhibited abrupt collapse and decreased volume (Fig. 8A). The amount of new bone at the border between the graft and recipient site

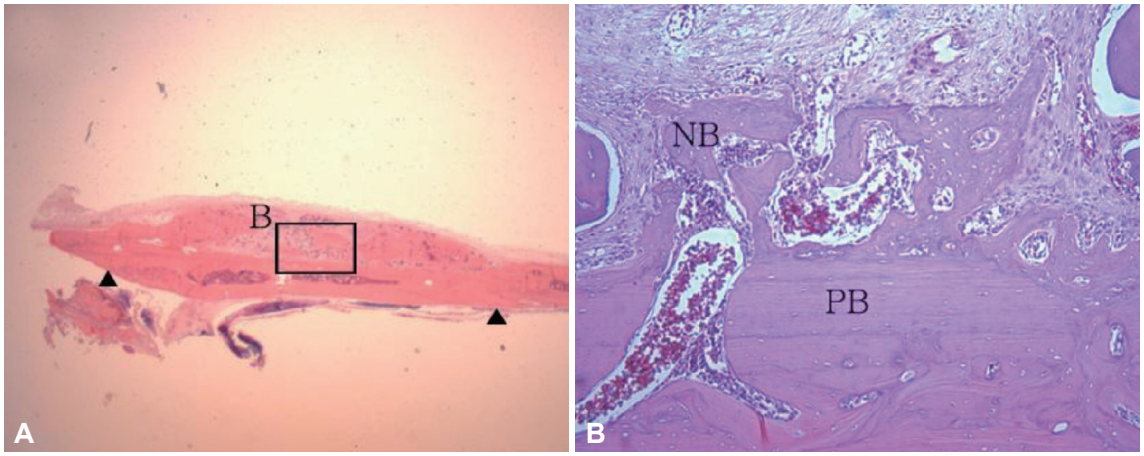

Figure 4. Representative photomicrographs of collagen sponge group at 8 weeks (B: region of interest in A; arrow head: grafted area margin, H-E stain; original magnification: A ×10; B ×100). PB: pre-existing bone, NB: new bone.

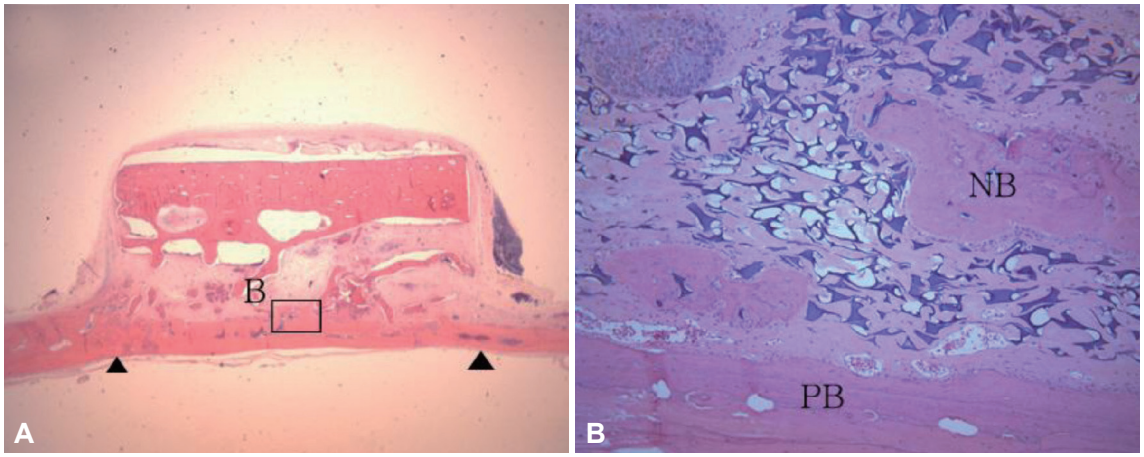

Figure 5. Representative photomicrographs of freeze-dried bone block group at 2 weeks (B and C: region of interests in A; arrowhead: grafted area margin, H-E stain; original magnification: A ×10; B ×100). PB: pre-existing bone, NB: new bone.

was increased, and adjacent bovine graft particles and new bone were in direct contact with each other (Fig. 8B). However, particles distant from the border area were surrounded by connective tissue (Fig. 8C).

Histomorphometric analysis

Total augmented area

There were statistically significant differences in the total augmented area between the three groups at week 2 postoperatively ($p<0.05$). The augmented area was significantly higher in the FDBB and DBBC groups than in the control group ($p<0.05$), and there was no significant difference between the FDBB and DBBC groups.

There were statistically significant differences between the three groups in the total augmented area at week 8 ($p<0.05$). The total augmented area was significantly higher in the FDBB and DBBC groups than in the control group, and the area was higher in the FDBB group than in the DBBC group ($p<0.05$).

When the total augmented areas were compared between

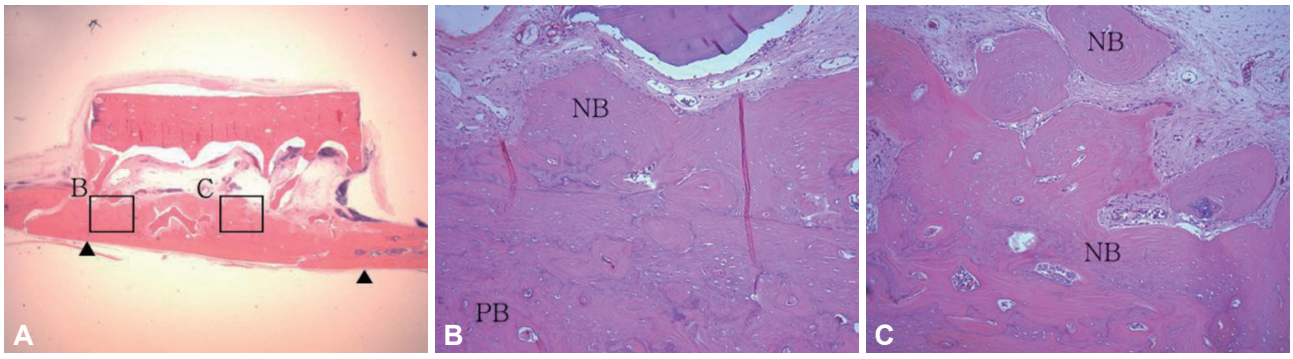

Figure 6. Representative photomicrographs of freeze-dried bone block group at 8 weeks (B and C: region of interests in A; arrowhead: grafted area margin, H-E stain; original magnification: A ×10; B and C ×100). PB: pre-existing bone, NB: new bone.

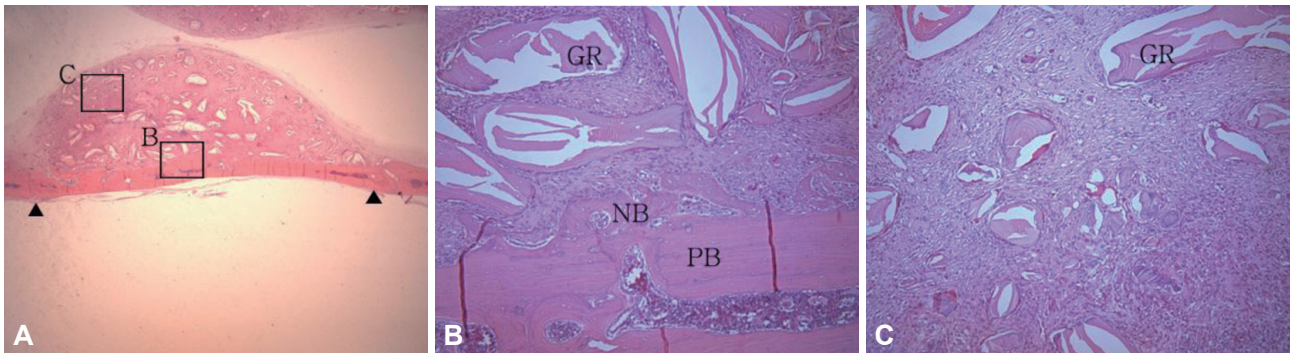

Figure 7. Representative photomicrographs of deproteinized bovine bone with collagen group at 2 weeks (B and C: region of interests in A; arrowhead: grafted area margin, H-E stain; original magnification: A ×10; B and C ×100). PB: pre-existing bone, NB: new bone, GR: graft material.

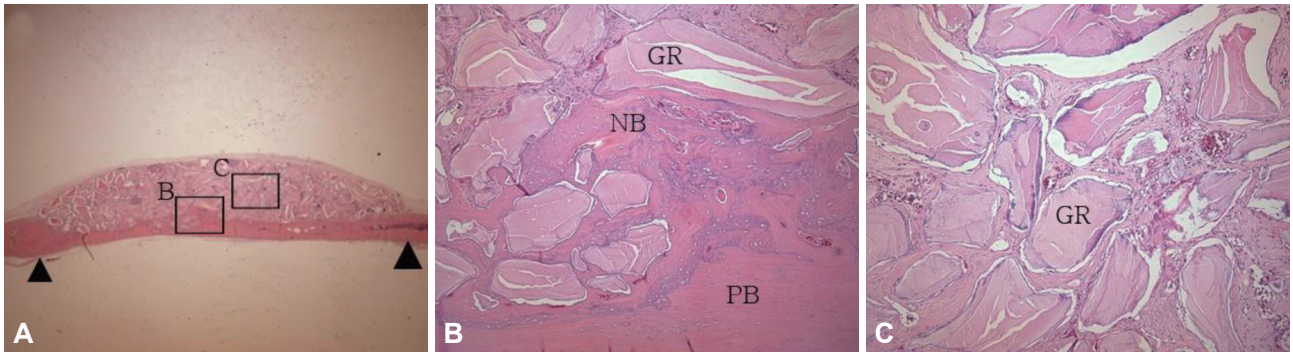

Figure 8. Representative photomicrographs of deproteinized bovine bone with collagen group at 8 weeks (B and C: region of interests in A; arrowhead: grafted area margin, H-E stain; original magnification: A ×10; B and C ×100). PB: pre-existing bone, NB: new bone, GR: graft material.

Table 2. Total augmented area

Group	2 weeks	8 weeks
CS	11.56±3.35	8.49±0.57
FDBB	58.60±8.43*	58.92±2.25*
DBBC	62.99±2.08*	32.80±2.15*†‡

Mean±standard deviation; mm^2, n=5. *statistically significant difference compared to CS group (p<0.05), †statistically significant difference compared to FDBB weeks group (p<0.05), ‡statistically significant difference compared to 2 weeks group (p<0.05). CS: collagen sponge, FDBB: freeze-dried bone block, DBBC: deproteinized bovine bone with collagen

Table 3. New bone area

Group	2 weeks	8 weeks
CS	0.08±0.02	0.18±0.03‡
FDBB	1.19±0.17*	4.63±1.02*‡
DBBC	0.38±0.10*†	2.06±0.56*†‡

Mean±standard deviation; mm^2, n=5. *statistically significant difference compared to CS group (p<0.05), †statistically significant difference compared to FDBB group (p<0.05), ‡statistically significant difference compared to 2 weeks group (p<0.05). CS: collagen sponge, FDBB: freeze-dried bone block, DBBC: deproteinized bovine bone with collagen

week 2 and week 8 postoperatively, the control and FDBB groups did not show any statistically significant differences, but the DBBC group showed a significantly decreased area at week 8 compared with week 2 (p<0.05) (Table 2).

New bone area

Statistically significant differences in the new bone area were observed in all three groups at week 2 and week 8 postoperatively. The new bone area was significantly greater in than the FDBB group than in the control and DBBC groups (p<0.05). Statistically significant differences in the new bone area were also observed in all three groups based on the postoperative duration. The new bone area at week 8 postoperatively was significantly greater than that at week 2 (Table 3).

Bone density

There were statistically significant differences between the three groups in bone density at week 2 postoperatively (p<0.05). In the multiple group comparisons, while there were statistically significant differences between the control and FDBB groups, and between the FDBB and DBBC groups, there was no statistically significant difference between the control and DBBC groups.

There were also statistically significant differences between the three groups in bone density at week 8 postoperatively (p<0.05). In the multiple group comparisons, the bone density in both the FDBB and DBBC groups were significantly higher than the bone density in the control group. There was no significant difference between the FDBB and DBBC groups.

Table 4. Bone density

Group	2 weeks	8 weeks
CS	0.77±0.35	2.19±0.43‡
FDBB	2.11±0.59*	7.86±1.78*‡
DBBC	0.65±0.22†	6.23±1.54*‡

Mean±standard deviation; %, n=5. *statistically significant difference compared to CS group (p<0.05), †statistically significant difference compared to 2 weeks group (p<0.05), ‡statistically significant difference compared to 2 weeks group (p<0.05). CS: collagen sponge, FDBB: freeze-dried bone block, DBBC: deproteinized bovine bone with collagen

When assessed according to postoperative duration, the bone density at week 8 was significantly higher than that observed at week 2 in all three groups (p<0.05) (Table 4).

DISCUSSION

Bone grafting is used to augment alveolar ridges showing severe horizontal and vertical resorption, and space maintenance using graft material is essential for a successful outcome. Although autogenous bone block grafting results in histologically and clinically significant bone formation, this approach is made difficult by donor site morbidity and the limited amount of collectable bone. Studies have investigated block-type graft materials using various alloplasts as a substitute for autogenous bone in order to improve the typically insufficient space maintenance provided by particle-type graft materials [15,23,24]. The objective of the current study was to histologically and histomorphometrically investigate the effects of onlay grafting of FDBB and DBB block with collagen on space maintenance and new bone formation in rat calvariums during the early postoperative recovery.

Because FDBB is rapidly frozen under high vacuum conditions and dehydrated, the immune response to FDBB is lessened, and the graft is stable at room temperature. The osteoinductivity and osteoconductivity provided by autogenous bone can also be expected, and the bone formation occurring via osteoconduction primarily serves as scaffolding. Meffert [25] reported that the cartilaginous matrix in human DFBAs at 6 months after bone grafting in the maxillary sinus resembled the bone found in FDBB.

DBBC is xenogenic bone obtained from cattle and is shaped similar to the human trabeculae; it also exhibits effective osteoconductivity [18]. Schlegel et al. [26] reported that while alveolar height decreased over time when pure autogenic bone was grafted in experimental animals, there was no change in height when a mixture of autogenous and xenogenic bones were gr-

afted. DBBC is easy to manipulate because it maintains a certain shape, which is mediated through the addition of collagen to the deproteinized bovine graft material. Although most clinical reports have been restricted to investigating its use in defects surrounded by bone housing, such as extraction sites, in this study, the effects of onlay grafts in block form on new bone formation were examined.

Factors influencing the prognosis of bone grafting include the size and shape of the oral defect, the use of autogenous bone graft, space maintenance, recovery time, immobilization of the graft material, and soft tissue adhesion. Among these parameters, space maintenance is an important factor that is associated closely with osteoconductivity, which is one mechanism of bone formation; therefore, an ideally sized and shaped bone regeneration cannot be expected if the space is lost. When graft material is absorbed too quickly relative to the time required for bone formation, the space is replaced by connective tissue rather than bone. Thus, the space, shape, and size of the augmentation must be maintained until adequate bone is generated by the graft material [27].

The material used in bone grafting receives stress generated by the surrounding tissue, such as bone housing or skin. Additional stress is especially generated when a large area requires augmentation. This stress can influence the space maintenance capability of the graft material and bone adhesion to the recipient site; therefore, an onlay rather than inlay experimental model is more effective when evaluating bone graft material for augmenting the alveolar ridge [28].

While FDBB has the properties of a solid, DBBC is subject to crumbling and disintegration. Considering that an onlay bone graft procedure was performed, stress from the tissue likely influenced the graft material. Because FDBB is solid, it can maintain its shape and is able to form adequate bone even when stressed by adjacent tissue. In contrast, DBSS, which is more pliable, is considered incapable of maintaining its shape. In the histological examination, DBBC showed minimal bone formation at week 2 postoperatively in the central area of the graft adjacent to the recipient site. The upper margin of the graft material, which was cylindrical at surgery, exhibited a circular, dome shape postoperatively. The graft showed signs of collapse, but the initial volume was largely maintained. At week 8, the amount of new bone in the center of the graft material adjacent to the recipient site increased, but the volume of the graft material greatly decreased from week 2 because of the collapse in the graft material.

Araujo et al. [29] found that the graft height and length were replaced by 30% and 50%, respectively, on histological examination performed 6 months after grafting DBB onto a canine alveolar ridge. In their study, DBB was immobilized using a barrier membrane and mini screws, and the recovery period was long at 6 months. In contrast, in the present study, the DBBC immobilization relied on suturing, and the recovery period was short at 8 weeks; hence, our histological findings may differ.

Based on the histomorphometric observations, the total augmented area in the DBBC group was smaller than that in the FDBB group, which is due likely to the inferior space maintenance provided by FDBB. Nevins et al. [19] histologically assessed periodontal regeneration after applying DBBC to the bone defect area. They reported clinically and histologically excellent results in bone defect area where the DBBC space was maintained. However, in defects where maintaining the space was difficult, a barrier membrane was required to maintain the space; based on this observation, the investigators drew a conclusion similar to that of this study.

The new bone area was also smaller in the DBBC group than in the FDBB group. Whether this result indicates that DBBC generates inferior bone formation requires further investigation. In the study by Araujo et al. [21], in which DBBC was immediately applied after extraction in dogs, the graft volume was well maintained without rapid resorption during the early recovery period of 8 weeks. We suspect that the contradiction between the results of the previous and current studies is due to differences between the inlay and onlay procedures. Potentially, use of the onlay technique created an unfavorable environment preventing the bone housing from being surrounded; the lessened new bone area may also be caused by insufficient space maintenance.

There was no difference in the bone density between the FDBB and DBBC groups. This may be caused by the smaller total augmented area in the DBBC group. The new bone area was also smaller in the DBBC group.

Sprague-Dawley rats were used as the experimental animal in this study. Because rats were used as recipients, both FDBB and DBBC are xenogenic bones; hence, bone formation completely depended on osteoconduction. Mellonig [6] conducted a clinical and histological evaluation of human bone defects repaired using allogenic bone. After grafting allogenic bone to the untreatable tooth and extracting the tooth after 6 months, the histological examination revealed greater bone formation than observed in this study, as well as regeneration of the periodontal ligament. Although the size and shape of the defect were more advantageous in the previous report, osteoinduction triggered by the allogenic graft may have contributed. In this study, the study period was shorter than what is the clinically accepted recovery period, which was done in order to evaluate those effects exclusive to the early postoperative stage. Therefore, a long-term study on the pattern of new bone formation and absorption of graft materials is needed. Further-

more, because DBBC used alone in onlay-type bone grafting showed limited space maintenance, additional studies are needed evaluating bone formation when a barrier membrane is used to maintain the existing bone and stabilize the adjacent surface.

In conclusion, the results of this study demonstrated that both FDBB and DBBC, when used for onlay bone grafting, caused bone formation through osteoconduction. These findings also suggest that DBBC has inferior ability to stimulate new bone formation and maintain the alveolar space compared with FDBB.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

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

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