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| Original Article |

The Effect of Human Freeze Dried Corticocancellous Block onlay Graft on Bone Formation in Rat Calvarium

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Abstract : The aim of this study was to investigate the effect of an onlay graft of human freeze dried corticocancellous bone block (FDBB) on bone formation and the added effects of collagen membrane (CM) in rat calvarium. Thirty male Sprague-Dawley rats were treated with either collagen sponge (CS), FDBB or FDBB with CM. FDBBs were placed on the calvarium surface with the CM covered or not. Rats were sacrificed at 2 and 8 weeks after surgery for histologic and histomorphometric analysis. At each period, total augmented area (TA; mm^2), new bone area (NB; mm^2), and bone density (BD; %) were measured. In the FDBB and the FDBB/CM group, new bone formation began at the lateral and inferior margins of the grafted block and projected into the central region of the recipient-graft interface. The cancellous portion of the graft underwent increased resorption with time. FDBB showed a significant decrease in the TA between 2 and 8 weeks ($p<0.05$), regardless of combined use of the CM. NB significantly increased in FDBB between 2 and 8 weeks ($p<0.05$), and the CM showed significant additional effect on new bone formation at 8 weeks ($p<0.05$). BD significantly increased in FDBB between 2 and 8 weeks ($p<0.05$). Within the limits of the present study, it was concluded that the maintenance of volume was achieved with onlay grafting of FDBB in early healing period to show new bone apposition onto the rat calvarial surface. In addition, using of CM improved new bone formation within in the grafted area.

Key words: *human freeze dried corticocancellous bone block, onlay graft, rat calvarium, collagen membrane, bone formation*

1. Introduction

Periodontal and endodontic lesions are most well known reasons for tooth extraction and might bring out severe destruction of alveolar ridge during the progression of the disease. Although dental implantation has become a reliable treatment option for replacement of missing teeth with good survival rate, it is a great challenge to install implants in the bone lack of vertical and horizontal volume. Various techniques have been suggested to augment a compromised alveolar ridge in dental fields including the use of bone graft materials with or without barrier membranes.[1-3]

Autogenous bone has been considered as the "gold standard" for bone graft materials because of its availability, biologic safety and ability to form new bone through three known mechanisms of bone regeneration: osteogenesis, osteoinduction and osteoconduction.[4] However, there have been drawbacks to the use of autogenous bone including increased operating times, donor site morbidity, limitations in amount, size and shape of available grafts, and the potential for intraoperative and postoperative complications.[5-7] To overcome the disadvantages associated with autogenous grafts, bone substitutes of allogenic, xenogenic or synthetic materials that depend mostly on the osteoconductive property in bone healing have been developed. Among these biomaterials, studies on the freeze dried bone allograft and demineralized freeze dried bone allograft in particulate form have been suggested as a viable alternative to autogenous graft.[8-10]

As far as onlay type grafting is planned to reconstruct a vertically resorbed ridge, augmentation with particulated bone alone, if not associated with additional device for space maintenance, has little capability of providing rigidity to

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withstand tension from the external compression forces exerted by soft tissue covering the area or provisional removable denture.[11,12] The use of block shaped graft material seemed to yield a considerable increase in vertical height of the ridge without the need in additional procedures, such as titanium mesh, a titanium-reinforced membrane, or miniplates that were used to protect the augmented volume.[13] Despite the successful results with human freeze dried bone block graft in orthopedic surgery for decades, there have been only a limited studies demonstrating the clinical application in alveolar ridge augmentation.[13,14] Some case reports have described adequate formation in the volume of reconstructed bone with human freeze dried bone block,[15,16] however, further comparative studies are required to guarantee the stable outcome and to evaluate the healing patterns of these blocks histologically.

It is debatable whether the block onlay graft should be covered by barrier membrane or not. Dongieux *et al*.[17] reported that there were no statistically significant differences in bone volume or histologic outcome between the healing of grafted onlay block covered by collagen membrane, e-PTFE membrane, or no membrane used in canine model. Many other studies, however, have supported the beneficial effects of membrane in onlay grafting procedure with autogenous block that the sites retained their augmented height by gradual bone fill underneath the membranes compared to the control site.[18,19] Still, there have been lack of researches about the necessity in barrier membrane to cover the human freeze dried bone block onlay graft.

Therefore, the aim of this study is to investigate the healing pattern and effects of the onlay grafting by human freeze dried corticocancellous bone block on new bone formation and secondly, effects of the covering graft materials with collagen membrane in a rat calvarium model using a histologic and histomorphometric analysis.

2. Materials and Methods

2.1 Animals

A total of 30 male Sprague-Dawley rats weighing about 200~300 g were used in this study. The animals were maintained in the cages kept in a room with 7-hr day/night cycles, an ambient temperature of 21°C, and a standard laboratory pellet diet. Animal selection and management, surgical procedures and preparations followed routine protocols approved by the Institutional Animal Care and Use Committee, Ewha Womans Medical Center, Seoul, Korea (ESM 09-0112). All institutional and national guidelines for the care and use of laboratory animals were followed.

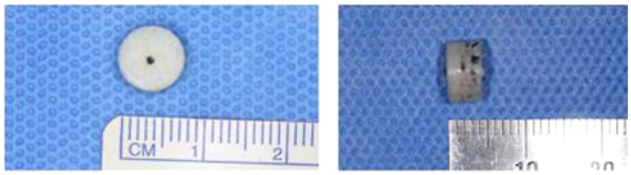

Figure 1. Preparation of disc shaped FDBB block.

2.2 Materials

Human freeze dried corticocancellous bone blocks (FDBB) (AlloBone, Osteo.in, Seoul, Korea) sterilized by low dose gamma irradiation were used in test groups (Table 1). The blocks were allowed to rehydrate for 30 minutes and the disc shaped preparation with the size of 4 mm in height and 8 mm in diameter were done (Fig 1). In FDBB covered with membrane group, collagen membrane (CM) composed of porcine collagen type I and III (BioGide®, Geistlich Biomaterials, Wolhusen, Switzerland) was used. Collagen sponge (CS) was placed in control site.

2.3 Surgical Procedures

Rats were anesthetized by an intramuscular injection (0.1 ml/10 g of a 3:2 solution of Zoletil : Xylazine). Full thickness flap was reflected to expose calvarial bone. With the use of round bur, the recipient site was subjected to six monocortical perforations with 1 mm width in each hole. Each graft material was placed on the parietal bone and stabilized with horizontal mattress sutures. After the placement, the periosteum was replaced at the original site and primary closure was done. Animals were divided into three groups with 10 animals per each and allowed to heal for 2 (n=5) or 8 (n=5) weeks. Each animal received one of the 3 types of materials ; collagen sponge (CS), freeze dried corticocancellous block bone (FDBB), freeze dried corticocancellous block bone/collagen membrane (FDBB/CM) (Table 1, Fig 2).

2.4 Histologic and Histomorphometric Analysis

The rats were sacrificed by CO_2 asphyxiation at 2 and 8 weeks after surgery. Tissue blocks including the materials with

Table 1. Experimental designs

Groups	Number of animals	
	2 weeks	8 weeks
CS	5	5
FDBB	5	5
FDBB/CM	5	5

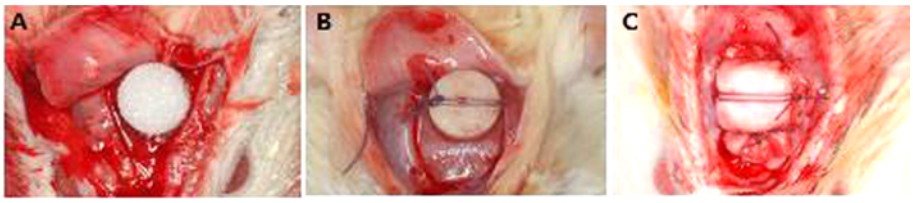

Figure 2. Placement of graft material on rat calvarium. (A) Collagen sponge (CS) in control group. (B) Human freeze dried corticocancellous bone blocks (FDBB) (AlloBone, Osteo.in, Seoul, Korea) group. (C) FDBB covered with collagen membrane (CM) (BioGide, Geistlich Biomaterials, Wolhusen, Switzerland) group.

underlying recent bone and surrounding soft tissue were harvested, fixed in 10% neutral formalin, decalcified and embedded in paraffin. The specimens were sagittally sectioned in 4 mm thickness and stained with hematoxylin-eosin (H-E). The most central sections from each block were selected for the histologic evaluation.

Histologic and histomorphometric analysis were performed using a videocamera on a light microscope (eclipse 50i, Nikon, Tokyo, Japan) and a computer-assisted automated image analysis system (Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA). Sections were examined at magnification of 10x and 100x.

The histomorphometric parameters were measured from the center of the sagittal section image using software in each specimen. The total augmented area was measured as the sum of all tissues beyond the cranial vault, including newly formed bone, residual graft materials (FDBB), and fibrovascular tissues. The new bone area was measured as the amount of newly formed bone shown within the total augmented area. The Bone density was calculated as the percentage of new bone area within the total augmentation area (Fig 3).

Defined standard of three histomorphometric parameters were as follows.

- Total augmented area (TA, mm^2) = Sum of all tissues beyond the cranial vault, including newly formed bone, residual graft materials, marrow, connective tissue, and vessels.
- New bone area (NB, mm^2) = the area of newly formed bone within the total augmented area.
- Bone density (BD, %) = (New bone area / Total augmented area) X 100

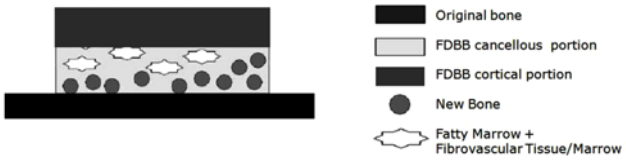

Figure 3. Schematic drawing of the parameters used in the histomorphometric analysis of the calvarial model.

2.5 Statistical Analysis

Mean values and standard deviations were calculated from the histomorphometric measurements for each parameter and group. For the comparisons among the treatment groups in each healing period, the Kruskal-Wallis test was used. The Mann-Whitney test was used to compare the difference between 2 and 8 weeks within each group ($p<0.05$).

3. Results

3.1 Clinical Observations

Wound healing was uneventful for all groups. No material exposure or other complications were observed at the surgical sites.

3.2 Histologic Observations

In the CS group, new bone was rarely formed and limitedly shown only at the surface of pre-existing calvarial bone (PB) at both 2 and 8 weeks. Collapse of the graft material was already

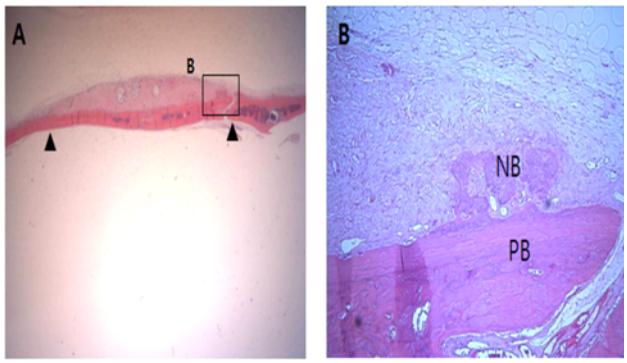

Figure 4. Representative photomicrographs of the CS group at 2 weeks (H-E stain). (A) Collapse of the graft material is seen between margins of the augmented area (arrowheads) (original magnification ×10). (B) High magnification of boxed region in view A. Limited new bone formation (NB) sprouted from the surface of pre-existing bone (PB) is shown (original magnification ×100).

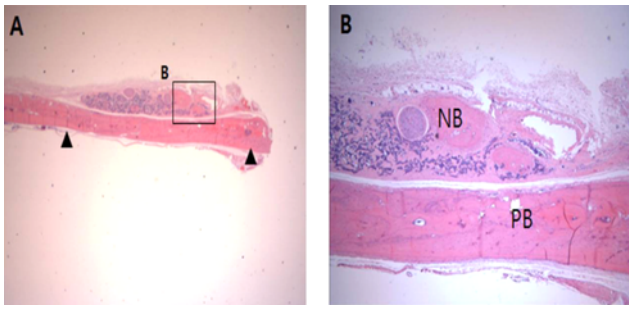

Figure 5. Representative photomicrographs of the CS group at 8 weeks (H-E stain). (A) Collapse of the material between margins of the augmented area (arrowheads) is more prominent from 2 weeks (original magnification ×10). (B) High magnification of boxed region in view A. Limited new bone formation (NB) adjacent to pre-existing bone (PB) is shown (original magnification ×100).

found at 2 weeks and the decrease of the volume was significant at 8 weeks (Fig 4, 5).

In the FDBB group at 2 weeks, newly formed bone was in direct contact with the PB surface and also limitedly projected into the space between the cancellous portions at lateral side of the graft material. The shape of the graft was still highly preserved and soft tissue covering the material was partly engaged into the lateral margin (Fig 6). At 8 weeks, the volume of the new bone apposition on the PB surface increased and

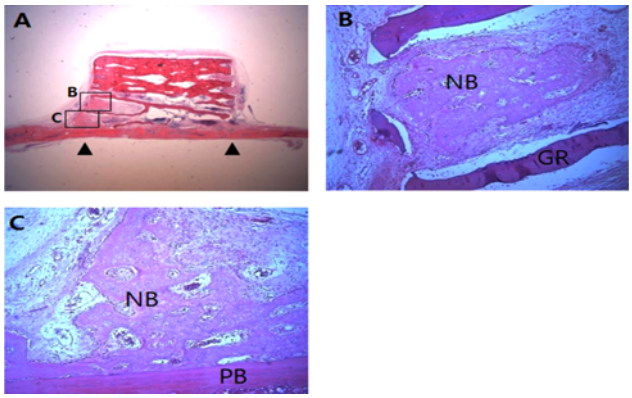

Figure 6. Representative photomicrographs of the FDBB group at 2 weeks (H-E stain). (A) Overall view shows graft material maintaining the space between augmented margins (arrowheads) (original magnification ×10). (B) High magnification of the boxed B region in view A. Newly formed woven bone (NB) is placed adjacent to the lateral side of the grafted space between the residual material (GR) (original magnification ×100). (C) High magnification of the boxed C region in view A. Woven bone (NB) in direct contact with the pre-existing bone surface (PB) and projecting into the connective tissue space near PB is shown (original magnification ×100).

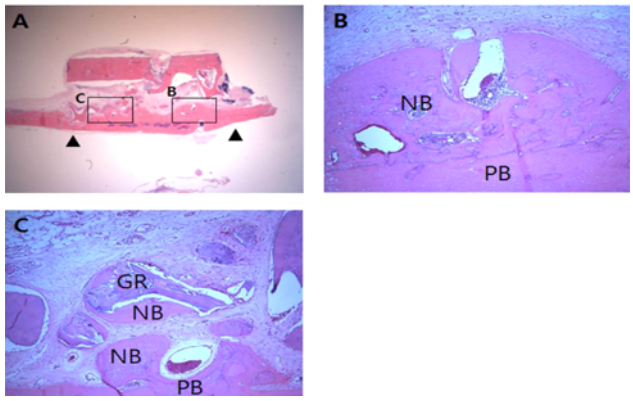

Figure 7. Representative photomicrographs of the FDBB group at 8 weeks (H-E stain). (A) Overall view shows the collapse in upper and lateral part of FDBB block between the augmented margins (arrowheads) with resorption in the middle cancellous portion and new bone partly occupying the resorbed space (original magnification ×10). (B) High magnification of the boxed B region in view A. Increased new bone (NB) directly connected to pre-existing bone (PB) is shown (original magnification ×100). (C) High magnification of the boxed C region in view A. Along with new bone (NB) formed on the surface of pre-existing bone (PB), it is also shown in direct contact with the surface of graft material (GR) (original magnification ×100).

new bone in direct contact with the resorbed graft particles was shown nearby. However, the lower and central portion of the material underwent the resorption and the volume was decreased (Fig 7).

In the FDBB/CM groups at 2 weeks, the shape and volume of the graft material was well maintained with dense fibrotic tissue of collagen membrane still covering the outer surface. No specific penetration of soft tissue inside the graft material was shown. Newly formed bone was in direct contact with and near the PB surface (Fig 8). At 8 weeks, new bone apposition increased on the PB surface and into the connective tissue space at the lower portion where the cancellous scaffold was resorbed. The volume of the graft material was significantly collapsed (Fig 9).

3.3 Histomorphometric Analysis

3.3.1 Total augmented area (TA)

Total augmented area (TA) of the FDBB and the FDBB/CM group was significantly elevated compared to CS group at both 2 and 8 weeks ($p<0.05$). In both the FDBB and FDBB/CM group, significant decrease in TA was shown between 2 and 8 weeks of healing ($p<0.05$). However, there was no significant difference between both grafted groups (FDBB, FDBB/CM) at 2 and 8 weeks (Table 2).

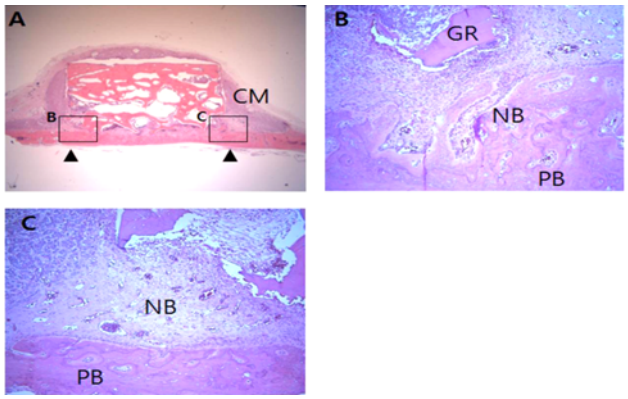

Figure 8. Representative photomicrographs of the FDBB/CM group at 2 weeks (H-E stain). (A) Overall view shows graft material with good maintenance of the original shape and dense fibrotic tissue of collagen membrane (CM) covering the surface (original magnification ×10). (B) High magnification of the boxed B in view A. Newly formed woven bone (NB) is being projected from pre-existing bone surface (PB) into connective tissue space where resorbed grafted material (GR) is partly found (original magnification ×100). (C) High magnification of the boxed C in view A. Newly formed woven bone (NB) is placed adjacent to pre-existing bone (PB) at augmented margin area (original magnification ×100).

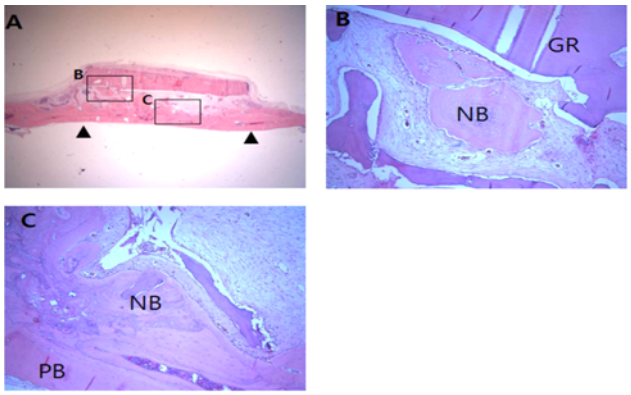

Figure 9. Representative photomicrographs of the FDBB/CM group at 8 weeks (H-E stain). (A) Overall view shows collapse of graft material in height with resorption of the cancellous portion. No specific invasion into FDBB scaffold by the soft tissue covering the outer surface is shown (original magnification ×10). (B) High magnification of the boxed B in view A. New bone (NB) between the residual graft material (GR) and pre-existing bone is seen (original magnification ×100). (C) High magnification of the boxed C in view A. Increased new bone (NB) apposition onto pre-existing calvarium surface appears beneath the remaining scaffold (original magnification ×100).

3.3.2 New bone area (NB)

Both grafted groups showed significant increase in new bone area (NB) compared to CS group at both 2 and 8 weeks (p<0.05). There was also statistically significant increase of NB at 8 weeks compared to 2 weeks in both grafted groups (p<0.05). The FDBB/CM group showed significantly greater NB compared to the FDBB group at 8 weeks of healing (p<0.05)(Table 3).

Table 2. Total augmented area of onlay graft in rat calvarium at 2 and 8 weeks (mean±SD; mm^2, n=5)

Group	2 weeks	8 weeks
CS	7.20±1.76	5.07±2.19
FDBB	29.66±1.28*	25.46±1.26*†
FDBB/CM	28.72±1.48*	26.63±1.13*†

*Statistically significant difference compared to the CS group (p<0.05).
†Statistically significant difference compared to the 2 weeks group (p<0.05).

Table 3. New bone area occupying total augmented graft in rat calvarium at 2 and 8 weeks (mean±SD; mm^2, n=5)

Group	2 weeks	8 weeks
CS	0.08±0.07	0.15±0.12
FDBB	1.51±0.34*	4.99±0.12*†
FDBB/CM	1.67±0.86*	6.07±0.53*†‡

* Statistically significant difference compared to the CS group (p<0.05).
† Statistically significant difference compared to the 2 weeks group (p<0.05).
‡ Statistically significant difference compared to the FDBB group (p<0.05).

3.3.3 Bone density (BD)

The FDBB and FDBB/CM groups showed significantly higher bone density (BD) compared to the CS group at 2 and 8 weeks (p<0.05). Both grafted groups showed statistically significant increase in BD at 8 weeks compared to 2 weeks (p<0.05). There was a tendency of higher bone density in the FDBB/CM group at 8 weeks compared to the FDBB group,

Table 4. Bone density within the augmented area at 2 and 8 weeks (mean±SD; %, n=5)

Group	2 weeks	8 weeks
CS	1.05±0.98	2.50±1.51
FDBB	4.57±1.90*	19.67±1.01*†
FDBB/CM	5.77±2.88*	23.20±2.86*†

* Statistically significant difference compared to the CS group (p<0.05).
† Statistically significant difference compared to the 2 weeks group (p<0.05).

although the statistical difference was insignificant (p=0.054) (Table 4).

4. Discussion

Reconstruction of severely localized ridge defects with block bone grafts is often an essential prerequisite for conventional prosthetic restoration or dental implant placement. Although autogenous bone grafts, in block or particulate form, remain the gold standard for ridge augmentation, donor site morbidity associated with block graft harvest has turned attention to the use of commercially available block graft materials.[15,16,20,21]

In this study, human freeze-dried corticocancellous bone blocks were used for onlay grafting procedure, which is considered to be more beneficial than inlay grafts to evaluate the effectiveness of materials for ridge augmentation of the ridge defects because the materials implanted onto the ridge defects would receive pressure from the periosteum, mucosa, or skin.[22]

From a histologic aspect, the collagen sponge collapsed significantly. Because collagen sponges can hold blood clots, they can be helpful in healing of extraction sockets. However, this material could not maintain its structural integrity as an onlay graft in this experiment. The FDBB onlay grafts integrated with pre-existing bone and showed new bone formation. New bone formation was evident especially at the recipient-graft interface and it was assumed that the recipient site provided the mesenchymal cells that invaded the graft and formed new bone. The volume still appeared to be highly preserved at 2 weeks. However, the graft underwent ncreased resorption at 8 weeks. These aspects were similar to the histological study of Pedora *et al*.[23] regarding autogenous onlay bone graft remodelling in rabbit calvarial bone. They presented that autogenous block grafts experienced a statistically significant loss of volume at 60 days compared with baseline and 20-day post-operative values.

While the loss of volume mostly came from the cancellous portion of the block, the cortical portion maintained its structural integrity until 8 weeks. Cortical thickness and density of donor bone were known to be factors that could possibly influence the resorption pattern.[23] Autogenous grafts with more cortical micro-architecture preserved the original bone volume more efficiently over time.[25,26] In this study, corticocancellous components provided the most predictable results; the cancellous portion allowed for vascular infiltration and led to the integration, while the cortical portion allowed for resistance to resorption.

According to the histologic results in our study, FDBB presented osteoconductive characteristics similar to those of the autografts. This was in agreement with an orthopedic study of allograft that showed the sequence of incorporation events were qualitatively similar to that for autografts.[27]

In our histomorphometric analysis, absorbable collagen membranes did not show additional effects on volume maintenance with time. The reason for the block bone resorption could be explained by the lack of vital bone forming cells in the graft following transplantation.[25,28] The resorption of the graft may, however, also be explained by external forces that act on the surgical site during healing. When a bone graft was placed on the surface of an edentulous ridge, the covering soft tissue flap must be stretched. This mobilization of the mucosa and periosteum may produce forces that acted on the graft leading to osteoclastic activity. [29] In this study, we used absorbable collagen membranes because of some disadvantages in the use of non-absorbable membranes including difficulty in handling and placement and risk of wound dehiscence and infection. Indeed, collagen membranes seem to be more ''tissue friendly'', easier to handle and thus show decreased risks of flap dehiscence and subsequent infections.[30] However, space-maintaining ability is a concern for the absorbable collagen membranes, especially when the mineralized block has sufficient rigidity. The absorbable collagen membrane could not give additional effect on FDBB blocks in volume maintenance against external forces in our study. This result coincided with a human study by Heberer *et al*.[31] suggesting that resorbable collagen membranes did not show explicit advantages compared with periosteal coverage of iliac bone grafts as the resorption rate showed no statistically significant differences.

Although the mechanical integrity is a weak point when used as a barrier, absorbable collagen membranes can prevent the non-bone-forming cells of the surrounding soft tissues from invading the bone graft, and thereby allowing the bone graft to be occupied by the bone-forming cells from the recipient bed only.[32] In this study, significantly more new bone formation was observed in the FDBB/CM group compared to the CS and FDBB group at 8 weeks. Taguchi *et al*.[33] reported that collagen membranes have osteoconductive capacity by promoting osteoblastic activity. Additionally, a study in a canine model revealed that the bioresorbable membrane enhanced bone regeneration, particularly when used in combination with graft materials.[34]

The local bone density has a prevailing influence on primary implant stability, which is an important determinant for implant success.[35] Although not statistically significant, the FDBB/CM group showed a higher bone density when compared to the FDBB group in this study (P=0.054). Because there were no significant differences in total augmented area between the FDBB and FDBB/CM groups, this result was attributed to

significantly more new bone formation in the FDBB/CM group compared to the FDBB group.

The manufacture recommendations in the healing of FDBB used in this study was 5 months for complete stabilization of the graft. Because of the short experimental periods, we could only examine early stage effects of the FDBB and collagen membrane. Further studies must be conducted in order to evaluate the aspects of later healing stage of the FDBB and long-term effects of collagen membrane on volume maintenance and new bone formation of the FDBB.

5. Conclusions

In the present study, we investigated the effects of onlay graft of human FDBB in bone formation and the effect of collagen membrane in rat calvarium. The findings in our study support the following conclusions:

FDBBs integrated with pre-existing calvarial bone (PB) and new bone formation adjacent to the graft material and host bone was observed. The volume of the grafted FDBBs appeared to be highly preserved at 2 weeks but showed a significant decrease at 8 weeks ($p<0.05$), regardless of combined use of collagen membrane. FDBBs showed significant increases in new bone formation and bone density between 2 and 8 weeks ($p<0.05$), and the addition of a collagen membrane showed significantly enhanced new bone formation at 8 weeks ($p<0.05$). Within the limits of the present study, it was concluded that the maintenance of volume was achieved with onlay grafting of FDBB in early healing period to show new bone apposition onto the rat calvarial surface. In addition, using of CM improved new bone formation within in the grafted area.

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Conflict of Interest: Hyejin Chung, Ji-Youn Hong, Gyu-Un Jung, and Eun-Kyoung Pang declare that they have no Conflict of Interest.

Ethical Statement: This study was approved by the Institutional Animal Care and Use Committee, Ewha Womans Medical Center

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